# Ammonium and nitrate uptake by *Eucalyptus nitens*

by

Trevor Paul Garnett, BSc. Hons (Adel.)

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy, University of Tasmania

December, 1996

## Declarations

This thesis contains no material which has been accepted for a degree or diploma by the University of Tasmania or any other institution. To the best of my knowledge and belief this thesis contains no material previously published or written by another person except where due acknowledgment is made in the text of the thesis.

Trevor P Garnett

This thesis may be made available for loan and limited copying in accordance with the Copyright Act 1968.

Trevor P Garnett

## Abstract

Low nitrogen availability commonly limits the growth of *Eucalyptus nitens* (Deane and Maiden) Maiden plantations in south-eastern Australia. However, until this study was undertaken little was known of the nitrogen uptake and assimilation processes of temperate eucalypt species like *E. nitens*.

In this study ammonium and nitrate uptake by young roots of solution cultured *E. nitens* were characterised under a variety of conditions using two different techniques: net uptake on a macro scale measured as depletion from solution, and net fluxes on a micro scale estimated from measurements of concentration gradients near root surfaces using microelectrodes.

Datasets taken from the literature were used to both validate the use of the depletion method for nitrogen, and to find the most appropriate method to use for the estimation of kinetic parameters from depletion datasets.

Ammonium and nitrate depletion from solution was characterised with respect to nitrogen source, pH, temperature, and N status. Ammonium uptake rates were consistently higher than nitrate uptake rates in all experiments. Uptake rates for both ammonium and nitrate were higher at pH 4 than at pH 6, and they were reduced to a similar extent with a decrease in temperature from 20°C to 10°C ($Q_{10}$ values of 1.3 to 1.9). For ammonium uptake, there was evidence for rapid adaptation of uptake processes (within 24 hrs) to changing temperature. Nitrogen status, which was correlated with relative growth rate (RGR), had unclear effects on uptake characteristics and highlighted deficiencies of measuring uptake by long term (up to 10 hours) nutrient depletion.

Ion selective electrodes were used in the MIFE (microelectrode ion flux estimation) technique to simultaneously measure ammonium, nitrate, and proton fluxes within the unstirred layer surrounding roots of *E. nitens* in solution culture. Measurements were within the region 20 to 50 mm from the tip of primary roots approximately 80 mm long. Within this region fluxes of ammonium, nitrate, and protons varied little, spatially or temporally. Under these conditions there was a consistent flux stoichiometry for ammonium: nitrate: and protons of 3.1: 1: -6.0. When ammonium and nitrate concentrations were both set at 100 μM there was no

inhibition of nitrate uptake by ammonium, but at concentrations of ammonium and nitrate in the range 200-1000 μM there was an apparent suppression of nitrate uptake. There was evidence for two mechanisms of ammonium uptake. The mechanism at low concentrations (<200 μM) showed saturation kinetics with a $K_m$ of 15 μM whereas at high concentrations (200-1000μM) an approximately linear increase in uptake occurred. Excision of the shoot brought about a rapid and dramatic reduction in fluxes of ammonium, nitrate, and protons.

The apparent preference shown here for ammonium over nitrate could be indicative of an adaptation by *E. nitens* to grow in cold, acidic, forest soils where ammonium is more readily available than nitrate. These results suggest that nitrogen uptake rates of *E. nitens* will be maximised if nitrogen is supplied predominantly in the ammonium form.

## Acknowledgments

The research described in this thesis was financially supported by the Cooperative Research Centre (CRC) for Temperate Hardwood Forestry. During this time I was supported by a PhD scholarship provided by the CRC.

Firstly, I would like to thank my primary supervisor, Philip Smethurst, for his support and guidance throughout this project. Philip has been an excellent supervisor, and without his help this work would not have come to fruition.

Ian Newman of the Physics Department kindly facilitated the use of the MIFE system. The knowledge and technical expertise of Sergey Shabala was indispensable during the MIFE work.

John Whittington has been a great friend and gave good counsel throughout this work. He also initiated my use of the MIFE system.

Robin Cromer graciously let me use the Ingestad growth units and his assistance was appreciated.

Paulina Teixeira, Jean-Noel Ruand, and Daniel Mendham have been constant sources of support, humour, encouragement and friendship.

I have been fortunate to have carried out this work in a productive and friendly environment. All my colleagues at the CRC for Temperate Hardwood Forestry and CSIRO Forestry and Forest Products have made this work extremely enjoyable. Specifically I would like to thank staff, past and present: Mike Battaglia, Neil Davidson, Peter Sands, Vin Patel, Don White, Libby Pinkard, Ann Lasala, Graham Osler, Linda Ballard, Jean Richmond, Brian Denton, Dale Worledge, Rabi Misra, Judy Sprent, Chris Beadle, and Phil West.

Brian Atwell was a gracious host during experiments carried out at Macquarie University.

I thank Mark Tester for starting me on a career in science and for being a constant source of inspiration. Thankyou to Robert Reid who taught me what to do in a laboratory, how to make computers do things, how to cook and why you should run.

My family have been supportive of this work and all other things in my life. My parents, Joyce and John Garnett, were always there with both love and financial support. Bruce Garnett and Jan Thompson supported this work financially near its completion.

Last, but by all means not least, my partner and friend, Jen McDonnell, has provided love and support throughout this work and has made me have fun outside of work.

## TABLE OF CONTENTS

# 1. Introduction

Nutrient levels naturally occurring in plantation forestry soils are usually not sufficient to sustain high productivity. In silviculture, as in agriculture, nitrogen (N) is one of the key nutrients limiting growth. To supplement naturally occurring N reserves in the soil, N fertilisation programs have been established in plantation forests throughout the world that are largely based on empirical experience and complicated by site and plant characteristics, and fertilisation practices (forms, rates, timing, placement) (Schönau and Herbert, 1989).

The need to maintain high rates of tree growth without over-fertilising demands that N fertiliser management becomes more site specific. An improved understanding of N supply, uptake, and utilisation should enhance our ability to manage N. The N uptake mechanisms of tree species have not been studied extensively and limits the use of uptake models that have been successfully used for describing P and K uptake by some species (Van Rees et al., 1990; Smethurst and Comerford, 1993).

*Eucalyptus nitens* plantations in south eastern Australia are commonly grown on sites that have previously supported either native forest, *Pinus radiata* plantations or pastures (Judd et al., 1996, Wang et al., 1996). Undisturbed, many of these soils have low rates of net N mineralisation, but, after harvesting and site preparation, stimulated mineralisation and reduced uptake lead to increased concentrations of $NH_4^+$ and $NO_3^-$ (Smethurst and Nambiar, 1990). Fertilisation practises further enhance N availability, with $NH_4^+$ and $NO_3^-$ levels in the soil solution maintaining levels greater than 1 mM for long periods (Hingston and Jones, 1985; Smethurst et al., 1997).

Although some eucalypt species grow very rapidly in response to increased N availability (Cromer et al., 1981; Birk and Turner, 1991) there is some doubt about the ability of eucalypts to fully utilise high concentrations of $NH_4^+$ and $NO_3^-$. For example, Moore and Keraitis (1971) compared growth of various *Eucalyptus* spp. on different proportions of $NH_4^+$ and $NO_3^-$, and found wide variation in the preference for either $NH_4^+$ or $NO_3^-$ between species from differing environments. Glasshouse experiments with seedlings of *E. diversicolor* and *E. globulus* showed that growth with $NO_3^-$ as the N source was depressed compared with $NH_4^+$ as the N source (Dell et al., 1991; Shedley et al., 1995). In a conifer, Jack pine, growth was depressed on

$NO_3^-$ compared with $NH_4^+$ and this depression was thought to be due to low $NO_3^-$ uptake rates (Lavoie et al., 1992). It seems likely that the apparent preference for $NH_4^+$ by some eucalypts is also due to lower rates of $NO_3^-$ uptake than those of $NH_4^+$. If this were the case for *E. nitens*, fertilisation practices that promote $NH_4^+$ instead of $NO_3^-$ as the form of N supply will maximise rates of N uptake and growth.

There have been few measurements of $NH_4^+$ and $NO_3^-$ absorption properties by tree roots, and apparently only one with a eucalypt species. Several non-eucalypt tree species have been shown to have preferences, in terms of higher uptake rates and sometimes higher affinity, for $NH_4^+$ as an N source (Scots pine, Boxman and Roelofs, 1988; maritime pine, Scheromm and Plassard, 1988; Norway spruce, Marschner et al., 1991; western hemlock, Knoepp et al., 1993; Douglas fir, Kamminga-Van Wijk and Prins, 1993; white spruce, Kronzucker et al., 1996). Previous work on N uptake by eucalypt roots carried out by Carrodus (1969), who measured $NH_4^+$ and $NO_3^-$ uptake by *E. obliqua,* found that uptake rates for $NH_4^+$ were higher than those for $NO_3^-$.

Environmental conditions have been shown to affect N uptake rates (e.g. soil pH, temperature, nutritional status), and in some cases differentially affect $NH_4^+$ and $NO_3^-$ uptake rates (Clarkson, 1985; Bloom, 1988; Engels and Marschner, 1995). Hence, these factors could result in a change in preference for $NH_4^+$ as an N source for *E. nitens*.

The simplest method of characterising uptake is to use kinetic parameters estimated from the depletion method (Claassen and Barber, 1974). However, the depletion technique has been applied in a variety of ways, none of which stands out as being the most appropriate. Additionally, in view of concerns about the depletion method for estimating kinetic parameters for N (Claassen and Barber, 1974), an alternative method would be advantageous. A novel method for measuring ion uptake by roots has recently been developed in which ion fluxes at the root surface in solution culture can be measured using microelectrodes (Newman et al., 1987; Henriksen et al., 1990, 1992; Kochian et al., 1992), and should be suitable for measuring uptake of both $NH_4^+$ and $NO_3^-$.

## *1.1 Objectives and Hypotheses*

### 1.1.1 Ammonium as the preferred N source for *E. nitens*

Based on what is known of the N nutrition of eucalypt species, together with that for other tree species from similar environments, it was hypothesised that *E. nitens* would show a preference for $NH_4^+$ as an N source. This preference for $NH_4^+$ should be apparent as higher maximum rates of uptake, a higher affinity (i.e. higher uptake at low concentrations), or lower concentrations below which uptake ceases, and exhibited over a wide range of environmental conditions.

Hence, this thesis addresses the following questions:

- Do *E. nitens* seedlings show a preference for $NH_4^+$ or $NO_3^-$ as an N source?
- If there is a preference, is it dependent on pH, temperature or nutritional status?

### 1.1.2 Methodological considerations

The first methodological objective of this thesis was to evaluate the use of the depletion method for N, and find an appropriate variation of this method for estimating N uptake parameters. The second methodological objective was to adapt the microelectrode method to simultaneously measure $NH_4^+$ and $NO_3^-$ fluxes at the root surface of *E. nitens* seedlings.

### 1.1.3 Kinetic parameters

Determine the $NH_4^+$ and $NO_3^-$ uptake kinetic parameters of *E. nitens* roots by the above two methods.

## *1.2 Outline of Chapters*

The thesis is divided into 7 chapters. This chapter is a general introduction in which the overall objectives are also described.

**Chapter 2** is a literature review on the topic of N uptake generally, and more specifically $NH_4^+$ and $NO_3^-$ uptake, by plant roots.

**Chapter 3** utilises N datasets from the literature to validate the use of the depletion method for N, and to recommend an appropriate variation to estimate kinetic parameters from depletion experiments.

In **Chapter 4** the depletion method was used to characterise $NH_4^+$ and $NO_3^-$ uptake by *E. nitens* under a range of conditions likely to be of importance when describing N uptake by plantation grown *E. nitens*. Aspects investigated were: N source, pH, temperature, growth rate and nutrient status.

In **Chapter 5** uptake of $NH_4^+$ and $NO_3^-$ by *E. nitens* was described by the microelectrode ion flux estimation (MIFE) technique, both to clarify results obtained using the depletion method and to investigate aspects that were not previously possible.

The results of the previous chapters are discussed in **Chapter 6** and compared in the context of the overall objectives.

Conclusions drawn from the research described in the thesis are presented in **Chapter 7**.

# 2. Literature review: Nitrogen uptake by plants

### 2.1.1 Nitrogen sources available to plants

Although $N_2$ is 78% of the earth's atmosphere, this form of N is not directly available to most plants. Only certain plant genera, via symbiotic relationship with micro-organisms, can directly harness this abundant source of N. The majority of plant species take up N from soil as $NH_4^+$ (ammonium) or $NO_3^-$ (nitrate) (Engels and Marschner, 1995). Roots of some species can take up low molecular weight forms of organic N either directly or via mycorrhizal symbioses (Chapin et al., 1993; Schulze et al., 1994; Jones and Darrah, 1994; Turnbull et al., 1995; Chapin, 1995), but these sources are only likely to be significant when rates of organic matter decomposition are low (Larsson, 1994).

### 2.1.2 Ammonium and nitrate in the soil

In unfertilised soils, the majority of $NH_4^+$ and $NO_3^-$ available to plants comes from decomposing organic matter in the soil. Organic N is transformed to inorganic N ($NH_4^+$ and $NO_3^-$) via mineralisation to produce $NH_4^+$, then nitrification to $NO_3^-$, with $NO_2^-$ as an intermediate form (Runge, 1981). Fertilisation results in higher concentrations of either or both $NH_4^+$and $NO_3^-$. Whereas uptake, leaching and denitrification are the main processes which decrease the concentrations of these N forms.

Environmental factors affect many of these processes, including rates of nitrification, and therefore the proportions of $NH_4^+$ and $NO_3^-$ available to plants. Warm soils generally have high nitrification rates which lead to $NO_3^-$ being predominant, whilst cold, waterlogged or acidic soils, tend to have low nitrification rates and $NH_4^+$ is predominant (Haynes and Goh, 1978).

Ammonium and $NO_3^-$ behave very differently in the soil. Ammonium is partly immobile because it is adsorbed to some extent on to cation exchange surfaces of organic matter and clay particles. In contrast, $NO_3^-$ is highly mobile in the soil and can be easily leached because it has very little interaction with the solid phase of the soil. The differences in mobility of $NH_4^+$ and $NO_3^-$ also affect the means by which these N

forms move to the root surface. Both diffusion and mass flow are important for $NH_4^+$, whereas $NO_3^-$ arrives at the root predominantly via mass flow (Barber, 1995).

## *2.2 Nutrient acquisition by roots*

Most mineral N and other nutrients entering a plant are taken up by the roots; only in high-ammonia environments is N uptake by shoots important (Marschner, 1995). Nutrient uptake is not uniform across all root surfaces of a plant. For annual species, a large proportion of the root surface actively takes up nutrients, whilst in established trees, the proportion of the total root surface that is actively absorbing nutrients is relatively unknown. Fine white roots of trees have high uptake rates, but older brown roots also appear to have some uptake activity and could make a major contribution to total uptake due to their large surface area (Eissenstat and Van Rees, 1994; Comerford et al., 1994).

Ammonium and $NO_3^-$ have relatively free access to the apoplast of the root, but they cannot move further into the root, i.e. past the endodermis, without moving across the plasma membrane into the symplast. Uptake can occur at either the epidermal cells and root hairs at the outer surfaces of the roots, or at the cortical cells. Root hairs are a common feature of young roots and can increase the root surface area by as much as 20 times (Glass, 1989).

## *2.3 Ammonium and nitrate uptake*

### 2.3.1 Energetics of uptake

To get access to the symplasm ions must pass across the plasma membrane which surrounds the symplasm. The plasma membrane is a lipid bilayer which has a hydrophilic outer surface and a hydrophobic (lipophilic) core. Due to their hydrophobic nature, hydrated ions such as $NH_4^+$ and $NO_3^-$ have very low permeability across the plasma membrane. For this reason, ions such as $NH_4^+$ and $NO_3^-$ require transporters to pass across the plasma membrane into the cytoplasm. Ion transporters are of two main types, those that facilitate transport across the plasma membrane down an electrochemical gradient, or those that harness an energy source to transport ions across the plasma membrane against an electrochemical gradient.

The electrochemical gradient of an ion across the plasma membrane is dependent on three main variables: the external concentration of that ion ($[ion]_o$), the cytoplasmic concentration of that ion ($[ion]_c$), and the electrical potential difference ($\Delta\Psi$) across the membrane.

At low concentrations at least, the electrochemical gradient for $NO_3^-$ is energetically uphill. This is due mainly to the $\Delta\Psi$ in most cases being negative to the value of approximately 100 mV (Nobel, 1983). Changes in $\Delta\Psi$ are dependent on factors such as nutritional history (Wang et al., 1994), but $\Delta\Psi$ is always negative. As $[NO_3^-]_o$ increases the concentration gradient can increase so that the chemical potential out weighs the electrical potential, but only if there is no concurrent rise in $[NO_3^-]_c$.

The energetics of $NH_4^+$ uptake are not as clear as those for $NO_3^-$ because for a long time it was unsure as to whether $NH_4^+$ did itself not cross membranes, or whether it was instead transported across as $NH_3$. Ammonium is a weak acid and exists in solution as a combination of $NH_4^+$, and the free base ammonia ($NH_3$), with proportions determined by pH ($pK_a = 9.25$). Ammonia, being neutral and small, has a very high permeability across lipid membranes (Kleiner, 1981; Ritchie, 1987). For this reason it was assumed that $NH_4^+$ was transported across the plasma membrane only as $NH_3$ (Kleiner, 1981). If this scenario was true, then $NH_4^+$ uptake would have shown strong dependence on pH (MacFarlane and Smith, 1982). Conversely, a number of studies have shown that $NH_4^+$ uptake does not show a strong dependence on pH and in fact can be reduced at high pH (McFarlane and Smith, 1982; Wang et al., 1993b). In agreement with this, there are a number of studies which have shown that $NH_4^+$ is transported across the plasma membrane of plant cells (Smith and Walker, 1978; Walker et al., 1979a, 1979b).

Ammonium being a positive ion and the $\Delta\Psi$ being negative means that transport across the plasma membrane will be electrically downhill. The only impediment to this downhill gradient would be high cytoplasmic concentrations of $NH_4^+$.

### 2.3.2 Intracellular nitrogen pools

#### *2.3.2.1 Cytoplasmic*

Ammonium concentrations within the cytoplasm have generally been thought of as very low. There were two reasons for this, firstly, because $NH_4^+$ at anything but very low concentrations was thought to be toxic (Mehrer and Mohr, 1989), and, secondly, because the capacity of the $NH_4^+$ assimilatory enzymes would assimilate any free $NH_4^+$ (Givan, 1979; Stewart et al., 1980). Ammonium toxicity relates to the equilibrium between $NH_4^+$ and $NH_3$. Ammonia rapidly permeates cell membranes and will remain in equilibrium across the plasma membrane. Accumulation of $NH_4^+$ would lead to dissociation producing $NH_3$ and $H^+$, the $NH_3$ diffusing out of the cytoplasm and the $H^+$ effectively disabling the proton gradient (Ryan and Walker, 1994). The activity of the main $NH_4^+$ assimilatory enzyme, glutamate synthetase, in roots is generally high and has a high affinity for $NH_4^+$ (Givan, 1979; Stewart et al., 1980). Based on this enzyme activity it has been assumed that any $NH_4^+$ in cells would be rapidly assimilated.

In spite of these earlier assumptions, recent studies have found cytoplasmic $NH_4^+$ concentrations in the mM range. Using $^{14}N$-nuclear magnetic resonance spectroscopy and maize roots, $NH_4^+$ levels in the cytoplasm have been found to be below 15 μM (Roberts and Pang, 1992), and in the low mM range (Lee and Ratcliffe, 1991). Using efflux analysis, $NH_4^+$ concentrations in the cytoplasm of rice grown on 1 mM were found to be 38 mM (Wang et al., 1993a), and for spruce roots grown at 1.5 mM, were found to be 33 mM (Kronzucker et al., 1995c).

These mM estimates seem to be at odds with the earlier assumptions of very low cytoplasmic $NH_4^+$ concentrations. It is not understood why mM concentrations of $NH_4^+$ do not lead to a disabling of the PMF. Kleiner (1981) hypothesised that membranes which had $NH_4^+$ transport systems would have reduced permeability to $NH_3$ to avoid such problems. It has also been suggested that $NH_4^+$ is compartmentalised within a rapidly exchanging pool in the cytoplasm in such a way as to not be distinguished by available detection methods (Ryan and Walker, 1994). Although not keeping $NH_4^+$ at very low concentrations, the role of assimilatory enzymes in maintaining lower concentrations of $NH_4^+$ is shown in experiments where

assimilatory enzymes are inhibited leading to 10-fold increases in cytoplasmic concentrations (Lee and Ratcliffe, 1991)

Unlike $NH_4^+$, $NO_3^-$ is a storage form of N and does not offer any problems of toxicity in the cytoplasm. Estimates of $NO_3^-$ concentration in the cytoplasm are also in the mM range, and for plants grown on 1-2 mM $NO_3^-$, cytoplasmic concentrations range from 4 mM in barley and spruce (King et al., 1992; Kronzucker et., al 1995a); up to 27-76 mM in onion (Macklon et al., 1990); 37 mM in barley (Siddiqi et al., 1991); and 10-20 mM in wheat (Devienne et al., 1994).

The cytoplasmic pools of $NH_4^+$ and $NO_3^-$ are both rapidly exchanging. The half time of exchange ($t_{1/2}$) for $NH_4^+$ ranges from ≈ 8 minutes for rice (Wang et al., 1993a), to ≈ 14 minutes for spruce (Kronzucker et al., 1995c). Estimates of cytoplasmic $t_{1/2}$ for $NO_3^-$ are shorter than for $NH_4^+$, and range from ≈ 4 minutes for barley (Lee and Clarkson, 1986); 4.6 minutes for wheat (Devienne et al., 1994); to ≈ 7 minutes for barley and spruce (Siddiqi et al., 1991; Kronzucker 1995a).

#### *2.3.2.2 Vacuolar*

The vacuole makes up 80-90 % of mature plant cells and is the main storage pool in plant cells (Clarkson and Luttge, 1984; Leigh and Wyn Jones, 1986). Although concentrations of $NH_4^+$ and $NO_3^-$ in the vacuole can be less than in the cytoplasm, the size of the vacuole compared with the cytoplasm results in the vacuole containing most of the cellular N. Concentrations of $NH_4^+$ in the vacuole have been estimated as being 4-11 mM in onion (Macklon et al., 1990); 8-15 mM in maize (Lee and Ratcliffe, 1991); and 3-6 mM in rice (Wang et al., 1993a). Estimates of $NO_3^-$ concentration in the vacuole are 22-31 mM in onion (Macklon et al., 1990); and 42 mM for barley (Walker et al., 1995).

Values of the vacuolar $t_{1/2}$ for $NH_4^+$ and $NO_3^-$ are much longer than those found for the cytoplasm. Estimates of $t_{1/2}$ for $NO_3^-$ are 16-21 hrs for barley (Belton et al., 1985); and 9.4 hrs for wheat (Devienne et al., 1994). For $NH_4^+$, only one estimate of the vacuolar $t_{1/2}$ could be found, and that was 8-22 hrs in onion (Macklon et al., 1990).

### 2.3.3 Mechanisms of uptake

#### *2.3.3.1 $NH_4^+$*

The nature of the low concentration, high affinity transport system (HATS), for $NH_4^+$ is dependent on whether uptake is energetically downhill or uphill. This point depends on the accuracy of estimates of both $[NH_4^+]_c$ and $\Delta\Psi$. It is generally thought that the HATS transporter for $NH_4^+$ is energetically downhill via an $NH_4^+$ uniport (Kleiner, 1981; Lee and Ratcliffe, 1991; Ullrich, 1992). Wang et al. (1994) measured $[NH_4^+]_c$ and $\Delta\Psi$ in rice and concluded that at low concentrations (dependent on previous $NH_4^+$ nutrition) $NH_4^+$ uptake was energetically uphill. They suggested that the transporter could be either an $NH_4^+/H^+$ symport, or a specific $NH_4^+$-ATPase. The former seems more likely considering that apart from $H^+$-ATPase and the $Ca^{++}$-ATPase, no other specific ATPases have been found in plants (Clarkson and Grignon, 1991).

Compartmentalisation of $NH_4^+$ within the cytoplasm, as proposed by Ryan and Walker (1994), would alleviate the apparent problems of toxicity and also change the energetics of uptake by reducing the effective cytoplasmic concentration of $NH_4^+$ making $NH_4^+$ uptake energetically downhill even at low external concentrations. There is, however, little evidence for this proposition.

At higher concentrations $NH_4^+$ uptake is via the low affinity transport system (LATS). Regardless of whether the HATS is energetically uphill or down hill, the $NH_4^+$ LATS is energetically down hill (Wang et al., 1994). The LATS could be mediated via either an electrogenic uniport, a specific $NH_4^+$ channel, or via $K^+$channels (Wang et al., 1994; Maathuis and Sanders, 1996). There are conflicting reports as to whether the LATS system for $NH_4^+$ is saturable or not. In soybeans the $NH_4^+$ LATS was found to be saturable (Joseph et al., 1975), however, in other species the $NH_4^+$ LATS did not saturate (Mäck and Tischner, 1994; Wang et al., 1993b; Kronzucker et al., 1996).

There is some evidence that the $NH_4^+$ HATS is inducible (Goyal and Huffaker, 1986; Morgan and Jackson, 1988a; Jackson and Volk, 1992; Mäck and Tischner, 1994), but Kronzucker et al. (1996), proposed that the supposed induction is actually a transient response to increased nutrient availability, and not induction at all. If any induction does occur, it is much less than that found for $NO_3^-$ uptake.

There have been some putative $NH_4^+$ transporters identified and even cloned from plant cells (Ninneman et al., 1994; Lauter et al., 1996), but their mechanism of transport were not determined.

*2.3.3.2 $NO_3^-$*

The $NO_3^-$ HATS operates against a steep electrochemical gradient. The changes in $\Delta\Psi$ due to nitrate, suggest a proton symport (McClure et al., 1990; Glass et al., 1992; Meharg and Blatt., 1995). Detailed electrophysiological investigations point to the transporter being a $2H^+$-$NO_3^-$ symport (Meharg and Blatt, 1995).

An important feature of the $NO_3^-$ HATS is it's apparent inducibility. Plants grown in the absence of $NO_3^-$ show very low $NO_3^-$ uptake rates when initially transferred to $NO_3^-$ containing solution, but within several hours of exposure to $NO_3^-$ there is a sigmoidal increase in $NO_3^-$ uptake rate (Henriksen and Spanswick, 1993). This induction has been noted in a large number of plant species (Minotti et al., 1969; Jackson et al., 1973; Goyal and Huffaker, 1986; Henriksen and Spanswick, 1993; Aslam et al., 1993). The occurrence of this induction has led to a distinction being made between the two HATS systems such that there are constitutive HATS (CHATS) and induced HATS (IHATS) for $NO_3^-$. The time of induction is thought to correspond to the time taken for synthesis of transport proteins (Siebricht et al., 1995).

There is also some evidence for another IHATS as well as the standard one. This other IHATS operates in a lower concentration range than the standard IHATS, at least in barley (Aslam et al., 1992) and spruce (Kronzucker et al., 1995d). It is possible that this system operates in other plants but the definition of results is not high enough for it to be distinguished.

It has been generally thought that the LATS system for $NO_3^-$ follows the pattern of LATS systems for other ions in being energetically downhill, possibly via anion channels (Glass, 1988). However, recent evidence suggests that the $NO_3^-$ LATS, like the HATS, is active via a $2H^+$-$NO_3^-$ symport (Glass et al., 1992; Tsay et al., 1993).

As with $NH_4^+$, there have been some putative $NO_3^-$ transporters identified (Tsay et al., 1993; Lauter et al., 1996).

### 2.3.4 Net uptake, influx and efflux

Net uptake of $NH_4^+$ and $NO_3^-$ is not simply influx, but the sum of both influx and efflux. Depending on conditions, efflux can be of the same magnitude as influx (Deane-Drummond and Glass, 1982; Lee and Clarkson, 1986; Morgan and Jackson, 1988b, 1989; Lee, 1993; Aslam et al., 1995). For $NH_4^+$, observed efflux is not simply a cycling of recently taken up $NH_4^+$, but also has a considerable component of endogenously produced $NH_4^+$ (Morgan and Jackson, 1988b, 1989). The mechanism of efflux could be via either ion channels (Marschner, 1995, Miller and Smith, 1996), or via proton mediated co-transporters (Marschner, 1995).

### 2.3.5 Assimilation and long distance transport

#### *2.3.5.1 Ammonium*

Ammonium is assimilated to organic N via the glutamine synthetase (GS) - glutamate synthase (GOGAT) system (Oaks, 1994). Ammonium assimilation produces at least one $H^+$ per $NH_4^+$ assimilated (Raven and Smith, 1976). Due to the problems of pH regulation, caused by the associated proton production, $NH_4^+$ assimilation mainly occurs in the roots (Raven and Smith, 1976). Depending on the nutritional regime some $NH_4^+$ can be found in the xylem, but this is a very small proportion of total $NH_4^+$ uptake (Allen et al., 1986; Kronzucker et al., 1995c). Phloem movement of $NH_4^+$ has been found, but this is also a very small proportion of total N uptake (Allen et al., 1986).

#### *2.3.5.2 Nitrate*

Assimilation of $NO_3^-$ to organic N involves conversion to $NH_4^+$ via nitrate reductase (NR) and nitrite reductase (NiR) (Oaks, 1994). Nitrate accumulation does not lead to toxicity as does $NH_4^+$ accumulation, so $NO_3^-$ can be assimilated in either the roots or the shoots. Nitrate assimilation produces almost 1 $OH^-$ per $NO_3^-$ assimilated, and, if assimilation occurs in the shoots, pH is maintained via the biochemical pH stat (Raven and Smith, 1976). The affinity of nitrite reductase greatly exceeds that of nitrate reductase so nitrite does not accumulate in these processes (Aguera 1990; Solomonson and Barber, 1990). These enzymes are thought to be

closely linked as very little free $NH_4^+$ accumulates in cells as a result of this assimilation process (Pilbeam and Kirkby, 1992).

Where $NO_3^-$ assimilation occurs in plants (roots or shoots) is highly variable, and dependent on plant type and environment (Fernandes and Rossiello, 1995). In woody plants, $NO_3^-$ assimilation was thought to occur mostly in the roots (Smirnoff and Stewart, 1985), but it is now clear that a large number of woody plant species can assimilate $NO_3^-$ in the shoots (Popp, 1993).

If all $NO_3^-$ is not assimilated in the root, $NO_3^-$ is transported in the xylem to the shoot. In plants where the majority of $NO_3^-$ assimilation is in the shoot, $NO_3^-$ transport in the xylem can almost equal the $NO_3^-$ uptake rate (van Beusichem et al., 1988; Peuke et al., 1996). As with $NH_4^+$, $NO_3^-$ transported to the shoots is assimilated there and is not present in the phloem in significant quantities (van Beusichem et al., 1988; Peuke et al., 1996).

## *2.4 Regulation of ammonium and nitrate uptake*

### 2.4.1 Demand for nutrients

The size of the assimilation and shoot transport sinks for a non-limiting nutrient source is under the control of shoot demand, which is mainly determined by the relative growth rate. The link between uptake and relative growth rate (RGR) was shown in growth experiments where uptake was determined by RGR over a wide range of external concentrations (Clement et al., 1978a, 1978b; Clarkson et al., 1986). A similar link has also been shown in split root experiments where uptake rates in one region of the root system increase to account for the removal of nutrients from another region (Drew and Saker, 1984; Burns, 1991).

Regardless of the long term control of uptake via growth, shorter term regulation of uptake occurs before an effect on RGR is noticeable. Nutrient deprivation has been shown to rapidly change potential uptake rates in a number of species (Lee, 1982; Drew et al., 1984; Lee and Rudge, 1986; Siddiqi et al., 1989). Clarkson (1985) suggested that changes in uptake kinetics are an early response to nutrient stress before RGR is affected.

### 2.4.2 What is the object of control?

There has been considerable discussion as to whether net uptake is regulated either via changes in influx (Lee, 1993; Muller et al., 1995), regulation of influx and efflux (Deane-Drummond, 1986), or only via regulation of efflux (Scaife, 1989). It appears likely that net uptake in the long term is under the control of changes in influx, whilst efflux regulates net uptake in the period (1-3 hours) before influx changes can occur (Lee, 1993).

There has been discussion as to whether efflux may indeed be an artefact of experimental design. Although efflux is a significant factor in experiments on $NH_4^+$ and $NO_3^-$ uptake in solution cultured plants, it may be of little consequence in soil growing plants. Macduff and Jackson (1994) argue that for plants growing in generally N limiting situations there would be little selective pressure for a capacity to regulate net uptake by changing efflux. Lee (1993), considering the energy economy aspect of maintaining the $H^+$ gradient during proton mediated co-transport, concluded that it would be advantageous to achieve a lower uptake rate by reducing influx rather than by promoting efflux.

### 2.4.3 What is the mechanism of control?

It has been proposed that the mechanism by which the shoot has control over net N uptake by the root is via the cycling of amino acids between the shoot and the roots (Cooper and Clarkson, 1989; Larrson et al., 1991). A surplus of amino acids in the shoot will be cycled back to the roots and reduce uptake. There is compelling evidence for amino acids affecting uptake by disrupting the synthesis of new carrier proteins (Muller and Touraine, 1992; Muller et al., 1995).

Together with this control by the shoot, it is argued, at least for $NO_3^-$, that cytoplasmic or vacuolar $NO_3^-$ levels have some regulatory control over uptake which is complementary to that of the amino acid control (Siddiqi et al., 1989; Mattsson et al., 1991; King et al., 1993; Kronzucker et al., 1995b).

Regulation of $NH_4^+$ uptake is somewhat simpler than for $NO_3^-$ because almost all $NH_4^+$ is assimilated in the root. For $NH_4^+$, feedback regulation appears to be caused by levels of $NH_4^+$ assimilates within the cytoplasm (Wang et al., 1993b; Kronzucker et al., 1995c).

### 2.4.4 Timescale of regulation

The timescale of uptake regulation is thought to be relatively rapid, e.g. Aribidopsis was found to adjust $NO_3^-$ uptake rates in 2-3 hours (Doddema and Otten, 1979). Plants subjected to various levels of N deprivation change uptake rates within 1 hour (Jackson and Volk, 1992). The induction of $NO_3^-$ uptake has been intensively studied, full induction occuring in 3-12 hours (Henriksen and Spanswick, 1993). Induction of $NO_3^-$ uptake is thought to involve synthesis of additional transporters which have half-times as short as 2-3 hours (Clarkson, 1986). The timescale of uptake regulation would be expected to be of similar scale to that for induction of the $NO_3^-$ uptake system.

# 3. Estimating kinetic parameters for N uptake using the depletion method

## *3.1 Introduction*

### 3.1.1 Kinetics to describe nutrient uptake

The concentration dependence of net nutrient uptake by roots (hereafter referred to simply as uptake unless otherwise stated) is described using kinetic parameters. Kinetics were first used to describe nutrient uptake by roots when Epstein and Hagan (1952) used Michaelis-Menten (M-M) style enzyme kinetic principles to describe radioactive tracer uptake by excised roots. In a way analogous to an enzyme catalysing the reaction between two substrates, proteins in the plasma membrane catalyse the transport of nutrients across the plasma membrane into the symplasm. In this way the transport of nutrients into the roots can be described by $V_{max}$ (enzyme $V_{max}$), the maximum rate of uptake, and $K_m$, the concentration at which uptake is half the maximum value. Following Epstein and Hagan (1952) kinetic parameters have become the accepted way of describing nutrient uptake by plant roots.

A typical concentration dependence of uptake curve is presented in Figure 3.1. At low concentration, i.e. less than 300 μM, there is a saturable uptake system corresponding to a HATS. The concentration of this curve can be described by the Michaelis-Menten relationship (Equation 3.1).

$$V = \frac{V_{max} + (C - C_{min})}{K_m \times (C - C_{min})} \qquad \text{Equation 3.1}$$

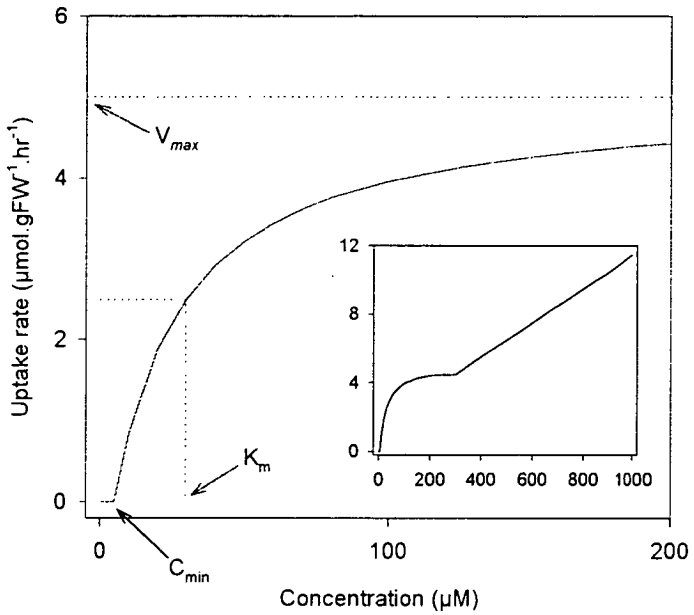
*where $V$ = uptake rate, $V_{max}$ = maximum uptake, $C$ = concentration, $K_m$ = concentration at half $V_{max}$, and $C_{min}$ = concentration where net uptake is zero.*

Equation 3.1 is revised in this case to include a $C_{min}$ term, which corresponds to the concentration below which net uptake no longer occurs (in this case 5 μM) (Nielsen and Barber, 1978; Drew et al., 1984). The $K_m$ in Figure 2.1. is 25 μM, and $V_{max}$ is 5 $\mu mol.g^{-1}.hr^{-1}$. The kinetic parameters, $K_m$ and $V_{max}$, only refer to the HATS, which in Figure 3.1 is below 300 μM. These parameters do not describe uptake above

this concentration. Above 300 µM in Figure 3.1, the LATS operates, which is shown in the inset.

The parameter $C_{min}$ is not widely used but is generally very low ($NH_4^+$ 1.5 µM, Marschner et al., 1991). However, $C_{min}$ is of importance when modelling nutrient uptake using process based models (Barber, 1995).

*Figure 3.1 A theoretical concentration versus uptake curve. Up to 300 µM uptake corresponds to the HATS and is described by the Michaelis-Menten function. As shown in the inset, above 300 µM uptake is due to the LATS and at physiologically relevant concentrations increases in a linear fashion with increasing concentration. (Adapted from Epstein, 1972).*




### 3.1.2 Range of values

The number of estimates of N uptake kinetic parameters found in the literature is extensive. A sample of these estimates is presented in Table 3.1. Further examples, including estimates from earlier literature can be found in Sands and Smethurst (1995). The range of estimated values for both $NH_4^+$ and $NO_3^-$ is large.

***Table 3.1. (Following page)*** *Kinetic parameters for N taken from the literature. The $V_{max}$ values were not always in units of µmol.g$^{-1}$.hr$^{-1}$ and where indicated (*) values were estimated. $V_{max}$ values are in terms of root fresh weight. Values presented are for plants previously exposed to $NH_4^+$ or $NO_3^-$ and only describe the standard HATS. Terms used to describe general methods and parameter estimation methods are described in the text.*

| | Plant | General Method | Parameter estimation method | $K_m$ (μM) | Vmax (μmol.g$^{-1}$.min$^{-1}$) | $C_{min}$ (μM) | Reference |
|---|---|---|---|---|---|---|---|
| (a) $NH_4^+$ | tomato | steady state | least squares | 6-12 | 9-19.8* | | Smart and Bloom (1988) |
| | barley | steady state | least squares | 15-28 | 9-9.6* | | Bloom (1985) |
| | carob | steady state | least squares | 10 | 2.3 | | Cruz et al. (1993) |
| | onions | 6 hr depletion | Claassen fit | 14 | 32-55* | 5 | Abbes et al. (1995) |
| | Spartina | 10-20 hr depletion | Claassen fit but forced Vmax | 1 | 1.3* | | Bradley and Morris (1990) |
| | Norway spruce | unidirectional flux 13-N | various including least squares | 20-40 | 1.9-2.4 | | Kronzucker et al. (1996) |
| | rice | unidirectional flux 13-N | least squares | 30-190 | 3-13 | | Wang et al. (1993b) |
| | spruce | 5 hr depletion | | | | 1.5 | Marschner et al. (1991) |
| | barley | steady state | Lineweaver/Burk | 153-166 | 3.7-8.3 | | Mäck and Tischner (1994) |
| | wheat | 8 hr depletion | not-specified | 50 | 3.5 | | Goyal and Huffaker (1986) |
| | Douglas fir | steady state | least squares | 8-14 | 0.7-0.9* | | Kamminga-Van Wijk and Prins, (1993) |
| (b) $NO_3^-$ | tomato | steady state | least squares | 34-54 | 18.6-30.6* | | Smart and Bloom (1988) |
| | barley | steady state | least squares | 7-187 | 3-15* | | Bloom (1985) |
| | maize | steady state | least squares | 224 | 10.7* | | Pace and McClure (1986) |
| | various catch crop species | 9 hr depletion | Hanes-Wolf | 5-36 | 9.8-35 | 10 | Laine et al. (1993) |
| | carob | steady state | least squares | 19 | 2.03 | | Cruz et al. (1993) |
| | onions | 6 hr depletion | Claassen fit | 11.9 | 14.3-29.7* | 4.54 | Abbes et al. (1995) |
| | barley | long term depletion | Lineweaver/Burk | 34-36 | 8 | | Aslam et al. (1992) |
| | spruce | unidirectional flux ($^{13}$N) | range including least squares | 112 | 0.7 | | Kronzucker et al. (1995 d) |
| | barley | unidirectional flux ($^{13}$N) | not specified | 30-79 | 1-9 | | Siddiqi et al. (1990) |
| | wheat | 8 hr depletion | not-specified | 27 | 2.6 | | Goyal and Huffaker (1986) |
| | barley | unidirectional flux ($^{13}$N) | least squares | 14 | 2.64* | | Lee and Drew (1986) |
| | lettuce | 7 hr depletion | visual inspection | 9.3 | 9.6* | 0.6 | Swiader and Freiji (1996) |
| | spruce | 5 hr depletion | | | | 22 | Marschner et al. (1991) |
| | corn, soybean sorghum, bromegrass | several day depletion | | | | 1.4-2.7 | Warncke and Barber (1974) |
| | spruce | not specified | least squares? | 200 | 18 | | Peuke and Tischner (1991) |

### 3.1.3 What different kinetics parameters reflect

Kinetic parameters are used to describe the concentration dependence of nutrient uptake. However, it is unclear what these parameters actually mean physiologically. Lee (1982) referred to $V_{max}$ and $K_m$ as being arbitrary measures of root performance. The value of $V_{max}$ is strongly influenced by nutrient supply during growth (Lee, 1982), with plants responding to nutrient deprivation generally by increasing $V_{max}$ (Glass, 1990). Changes in $V_{max}$ are thought to be mediated by changes in the number of transport proteins (Drew et al., 1984; Clarkson, 1985). The value of $K_m$ does not appear to be effected in the same way as $V_{max}$. Prior nutrient status has varied effects on $K_m$, with large effects on $K_m$ rarely seen (Lee, 1982), but more recent studies have shown large effects (but still smaller than that for $V_{max}$) for both barley (Siddiqi et al., 1990) and rice (Wang et al., 1993b). The mechanisms by which $K_m$ changes are unclear. Meharg and Blatt (1995) highlighted the effects of changes in $\Delta\Psi$ on observed $K_m$ values. As nutritional status has been shown to affect $\Delta\Psi$ (Wang et al., 1994), changes in $K_m$ could be due to this factor. It has been suggested that the apparent $K_m$ for a nutrient, may reflect the concentration of that nutrient found at the soil-root interface (Smart and Bloom, 1993).

### 3.1.4 Kinetics estimation

A variety of methods have been developed for estimating uptake parameters. In one method, the "tea bag" method (Epstein et al., 1963), the rate of accumulation of radioactive tracer in excised roots is measured at various concentrations in solution. This method results in the rate of uptake expressed as a function of concentration which, at low concentrations, follows M-M style kinetics. The use of excised roots may not reflect uptake by an intact plant and the same basic principles have been applied to measure uptake in roots of intact plants (Lee and Drew, 1986; Siddiqi et al., 1990; Wang et al., 1993b; Kronzucker et al., 1995d, 1996). This technique requires a radioactive isotope of the nutrient of interest, but there is no easily available isotope suitable for N. The stable isotope of N ($^{15}N$) is not generally suitable for short term measurements (Clarkson, 1986; Lee and Clarkson, 1986; Clarkson et al., 1996) and the radioactive isotope, $^{13}N$, has such a short half-life ($\approx$ 10 min) that it requires the close proximity of a cyclotron (Clarkson et al., 1996).

In another method, uptake is measured as the short-term decrease in concentration in solution at different starting concentrations. The extent of the depletion at each concentration is usually the minimal detectable concentration change, which is generally less than 10 percent of the starting concentration. For example, uptake at 100μM will be measured as the depletion from 100μm down to 90μM. By repeating this for a range of concentrations, uptake rate (V) can be plotted as a function of concentration (C), i.e. a V(C) function. Variations of this technique have been developed by a number of workers (Rao and Rains, 1976; Bloom, 1989; Raman et al., 1995a, 1995b). A long-term version of this technique has also been used by others (Wild et al., 1979; Mullins and Edwards, 1989), where groups of plants are grown at the same concentration at which uptake is measured. In this way each point on a V(C) function is an uptake rate measured for plants grown at that concentration.

For each V(C) plot, the methods described above require a number of different starting concentrations, and some of these methods also require a number of different root samples at each concentration. Claassen and Barber (1974) developed an alternative method where, for a single V(C) function, only one root sample and one starting concentration was required. Plants were placed in a known concentration and the solution sampled until depletion was complete. The rate of concentration decrease at a specific concentration indicated the uptake rate at that concentration. The key advantage of this method is that only one plant per replicate needs to be used to construct a V(C) function, and thereby estimate kinetics. As only the solution concentration needs to be measured, no uptake tracer is required. The Claassen and Barber method, hereafter referred to as the depletion method, has been widely used for estimating uptake kinetics (Bhat, 1983; Goyal and Huffaker, 1986; Bradley and Morris, 1990; Laine et al., 1993; see Table 3.1).

### 3.1.5 Depletion methodology

The depletion method involves two stages of data analysis: (i) estimating the rates of uptake at various concentrations, and from these data, (ii) estimating the M-M uptake kinetic parameters. In the first stage, a function can be fitted to the concentration data in relation to time (t) to, firstly, smooth out experimental errors in the C(t) data, and secondly, to provide an equation for these data for which the derivative (dC/dt) can be obtained to, in turn, calculate net rates of uptake at various

concentrations, i.e. V(C). In the second stage, the M-M function is fitted to these V(C) data to estimate the kinetic parameters $K_m$ and $V_{max}$. Although this two stage approach is commonly used, the preferred method of Claassen and Barber (1974) combined these two stages into one non-linear fitting operation. In this application of the depletion method the C(t) data were fitted to the first derivative of a modified version of the M-M rate equation (Equation 3.2).

$$\frac{dC}{dt} = -L\left[\frac{V_{max}C/v}{K_m + C/v} - E\right] \qquad \text{(Equation 3.2)}$$

*where C = concentration, L = root length, $V_{max}$ = maximum uptake rate, Km = concentration at half maximum uptake rate, v = solution volume, and E = efflux rate.*

The one-step Claassen and Barber method for data analysis has been widely used but many alternative methods have also been developed. The main reason for the development of these other techniques appears to have been the lack of robustness of the Claassen and Barber method. Unless there are good initial estimates of what parameter values might be, and the C(t) data have a close relationship to the theoretical M-M relationship, the fitting method does not work (Van Rees, 1994).

Descriptions of C(t) data have included various order polynomials and intrinsically non-linear functions. These functions were all attempts to give the closest possible fit to trends in the experimental data. Unlike the Claassen and Barber one step method, none of the C(t) functions have any physiological significance. Although the sole purpose of these functions is to accurately describe the C(t) data in a differentiable equation, there has been some inconsistency about which functions should be used, and justifications of choice have rarely been made.

Some of the most popular methods for estimating kinetic parameters from the V(C) data have been Lineweaver-Burke plots and variations on this theme such as the Hanes-Woolf and Hoftsee plots. These techniques, which were developed before personal computers were readily available, used linearised transformations of the data to simplify calculations. However, if improperly used, these transformations can lead to biased or unreliable estimates of kinetic parameters (Ritchie and Pravan, 1996). These authors found that the most reliable method of parameter estimation was direct fitting of the V(C) data to the M-M formulation (Equation 3.5) using a non-linear

curve fitting package. This method is simple, robust, and gives the most statistically accurate parameter estimates.

Van Rees (1994) compared some of the different methods for fitting C(t) data and concluded that there were only small differences in estimated parameters due to different estimating methods. Unfortunately, in the datasets used there was not always full depletion to $C_{min}$, and there were few points around the concentration expected for the $K_m$. Another potential problem was that the Lineweaver-Burk-style plots used for estimating kinetic parameters were unweighted. Based on the unreliability of this method of parameter estimation (Ritchie and Pravan, 1996), differences between the C(t) fitting functions may have been obscured in the parameter estimation process.

### 3.1.6 Depletion and nitrogen

The depletion method was initially developed for $K^+$ uptake by corn (Claassen and Barber, 1974) and they commented that their model (Equation 3.2) was not appropriate for nitrogen. They suggested a different model may be necessary to describe N uptake by corn. Morris (1980) justified the use of the M-M relationship for describing N uptake by plants because, in a long-term growth experiment, M-M kinetics satisfactorily described long-term uptake. However, this was a justification of the use of M-M kinetics to describe N uptake by plant roots generally and not a justification based on conditions found in depletion experiments. Regardless of the adequacy of the M-M relationship to describe N uptake in depletion experiments, the depletion method has been used to estimate N uptake kinetics for a wide range of species (Bhat, 1983; Goyal and Huffaker, 1986; Bradley and Morris, 1990; Laine et al., 1993; Swiader and Freiji, 1996; see Table 3.1).

### 3.1.7 Objectives

Doubts about the methods of describing the C(t) data and of assuming M-M-like uptake kinetics for N indicated a need to evaluate these aspects if depletion methods were to be used to quantify N uptake kinetics in eucalypt roots.

The objectives of this chapter were to:

- evaluate alternative C(t) fitting functions and select the most appropriate one,

- determine the most appropriate form of the M-M function to use in kinetic parameter estimation, and
- evaluate the use of the depletion method for estimating kinetic parameters for N depletion experiments

## *3.2 Methods*

### 3.2.1 Datasets

Data were taken from 3 papers where the depletion method had been used to estimate kinetic parameters for N uptake (Table 3.2). Claassen and Barber's (1974) $K^+$ dataset was also used as a comparison. The C(t) data were for individual plants, or in some cases groups of plants.

***Table 3.2.*** *Data sources for evaluating methods of parameter estimation for $NO_3^-$ and $K^+$ uptake using the depletion method. Data were read from figures except for Claassen and Barber 1974 where they were tabulated.*

| Reference | Ion | Source in Reference |
|---|---|---|
| Edwards and Barber, 1976 | $NO_3^-$ | Figure 1. |
| Laine et al, 1993 | $NO_3^-$ | Figure 1. a. Replicate 3 |
| Swiader and Freiji, 1996 | $NO_3^-$ | Figure 1, IC labelled data |
| Claassen and Barber, 1974 | $K^+$ | Table 1 |

### 3.2.2 C(t) functions and preparation of V(C) data

Three functions were used to describe the depletion curves: (1) A logistic function used by Wild et al., (1979):

$$y = a + \frac{c}{1 + e^{b(t-f)}} \qquad \text{(Equation 3.3)}$$

*where $t$ = time, $a$, $b$, $c$, and $f$ are constants and $e$ is the base of natural logarithm.*

(2) A rational function used by Bhat et al., (1981):

$$y = a + \frac{b + ct}{1 + dt + et^2} \qquad \text{(Equation 3.4)}$$

*where t = time and a, b, c, and d are constants.*

(3) Fifth degree (5th order) polynomials were also fitted to the data. Statistically, the use of a 5° polynomial and not a lower order polynomial is generally not justifiable (Goyal and Huffaker, 1986) but it has the advantage that it can fit almost any shape of depletion data. Fitting was carried out using either the polynomial regression or non-linear curve fitting components of Sigma Plot (Jandel Corporation, US). The fitted functions were differentiated and the slope calculated at each data point. This slope was taken as the uptake rate at the concentration of the data point. As all experimental parameters required (root weight, area, or length and solution volume) were not generally available the uptake rates were presented in relative terms. Because these estimates were intended only for comparisons between the different techniques, the use of relative values was justified.

### 3.2.3 Estimation of kinetic parameters

The V(C) data were described by the M-M equation (Equation 3.5) using the non-linear fitting function of the computer package Sigma Plot (Jandel Corporation, US).

$$V = \frac{V_{\max} + C}{K_m \times C} \qquad \text{(Equation 3.5)}$$

*where V = uptake rate, $V_{max}$ = maximum uptake rate, C = concentration, and Km = concentration at half $V_{max}$.*

Equation 3.5 was fitted to unmodified V(C) data described above, and to modified V(C) data for which high and low concentrations points were removed. The points removed were deemed to be artefacts of the C(t) fitting process and resulted in V(C) values at high and low concentration that could clearly not be described by the M-M relationship.

In some cases the fit of the standard M-M function (Equation 3.5) can be improved by incorporating $C_{min}$ in the function, as in Equation 3.1 (Nielsen and Barber, 1978; Drew et al., 1984) so this was the second function fitted to the data.

The value of $V_{max}$ has been estimated as being the slope of the initial section of the depletion curve before a clear reduction in uptake rate takes place (Bradley and Morris, 1990; Swiader and Freiji, 1996). Hence, $V_{max}$ was estimated according to this third method as the slope of a linear regression of the initial part of the depletion curves. This estimate of $V_{max}$ was then incorporated into the M-M relationship as an independent variable for estimating $K_m$ and $C_{min}$ (Bradley and Morris, 1990).

## *3.3 Results*

### 3.3.1 C(t) functions

For the four datasets, the three functions (5° polynomial, Bhat, and Wild) closely fitted the full range of the C(t) data (Figure 3.2). However, the C(t) fits were not identical. Differences between the curve fitting methods become clearer when the V(C) data were calculated (Figure 3.3). These differences were greatest near maximum uptake rates and at high concentrations. For each dataset, the Wild function had the highest uptake rates and at the highest concentrations gave the lowest uptake rates. At high concentrations, the 5° polynomial fit provided the highest estimated rates of uptake.

***Figure 3.2.*** *Depletion data from different sources fitted using three different fitting functions. Datasets are (a.) Edwards and Barber, 1976, (b.) Laine et al., 1993, (c.) Swiader and Freiji, 1996, and (d.) Claassen and Barber, 1974. Datapoints are presented as symbols and fitted functions as lines.*

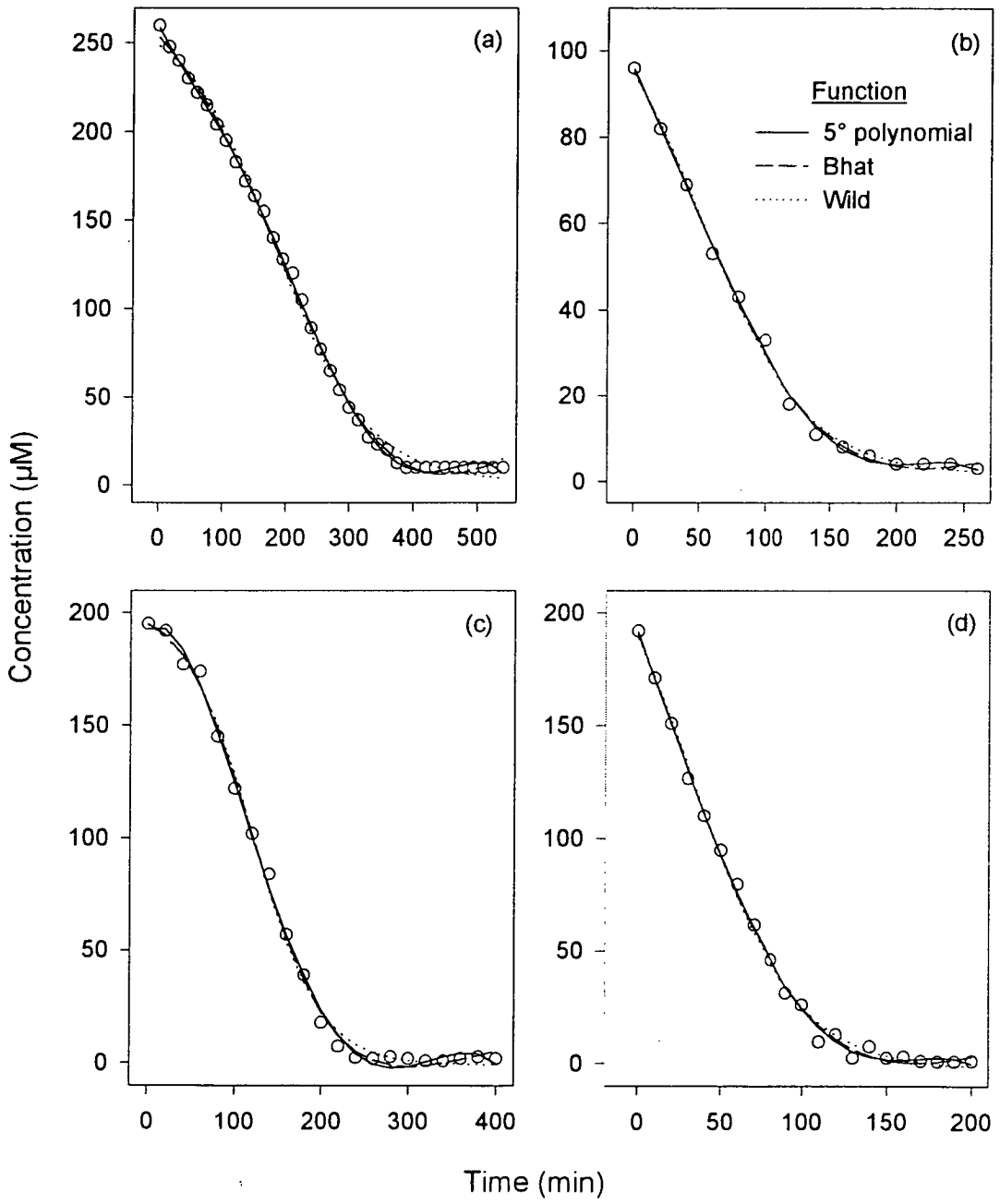


***Figure 3.3.*** *The V(C) data produced for four datasets using the three types of C(t) fitting functions: 5° polynomial, Bhat function, and Wild function. The V(C) data are represented as symbols whilst the lines are produced from fitting the standard M-M function to these data. Datasets are as in Figure 3.2.*

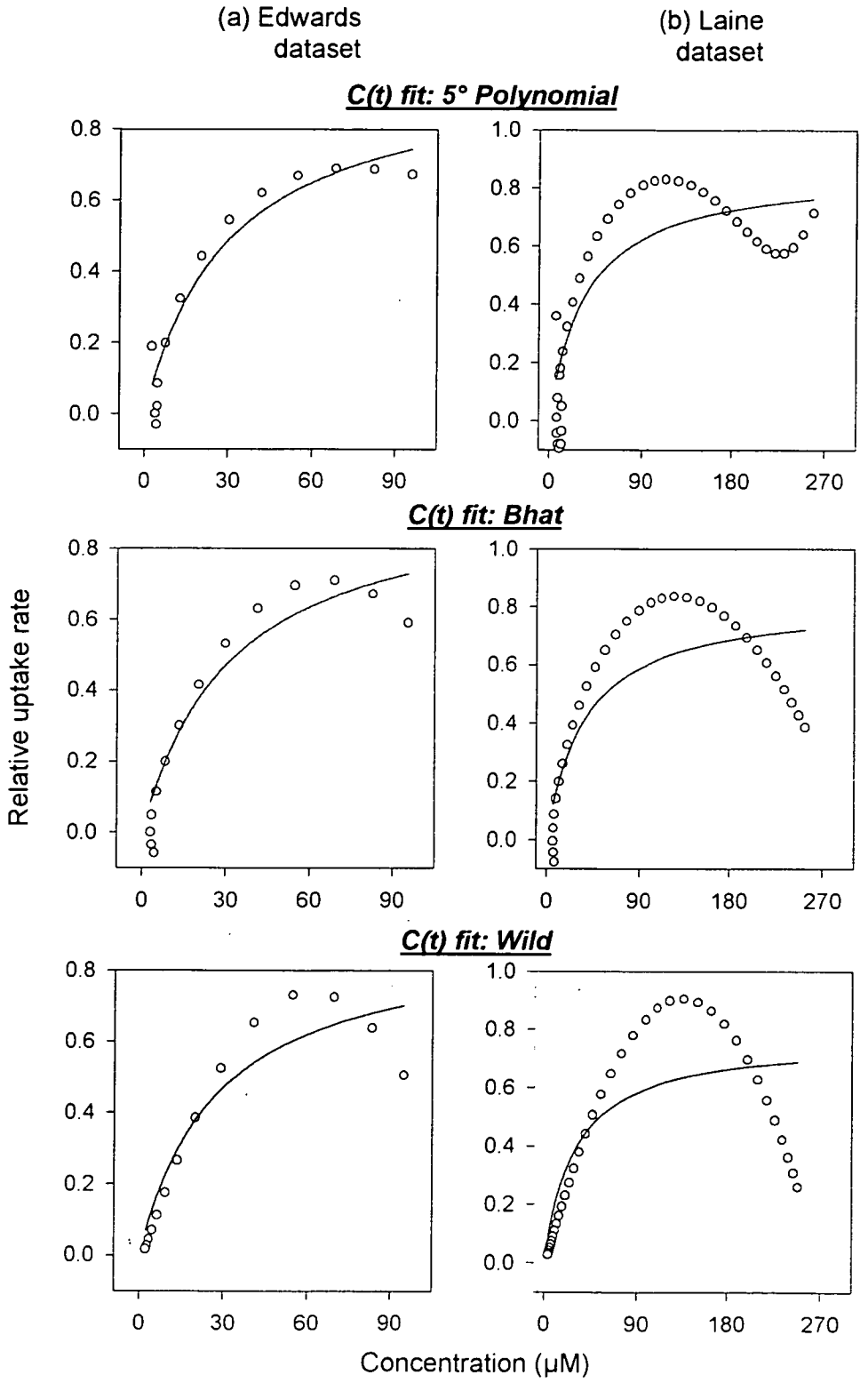
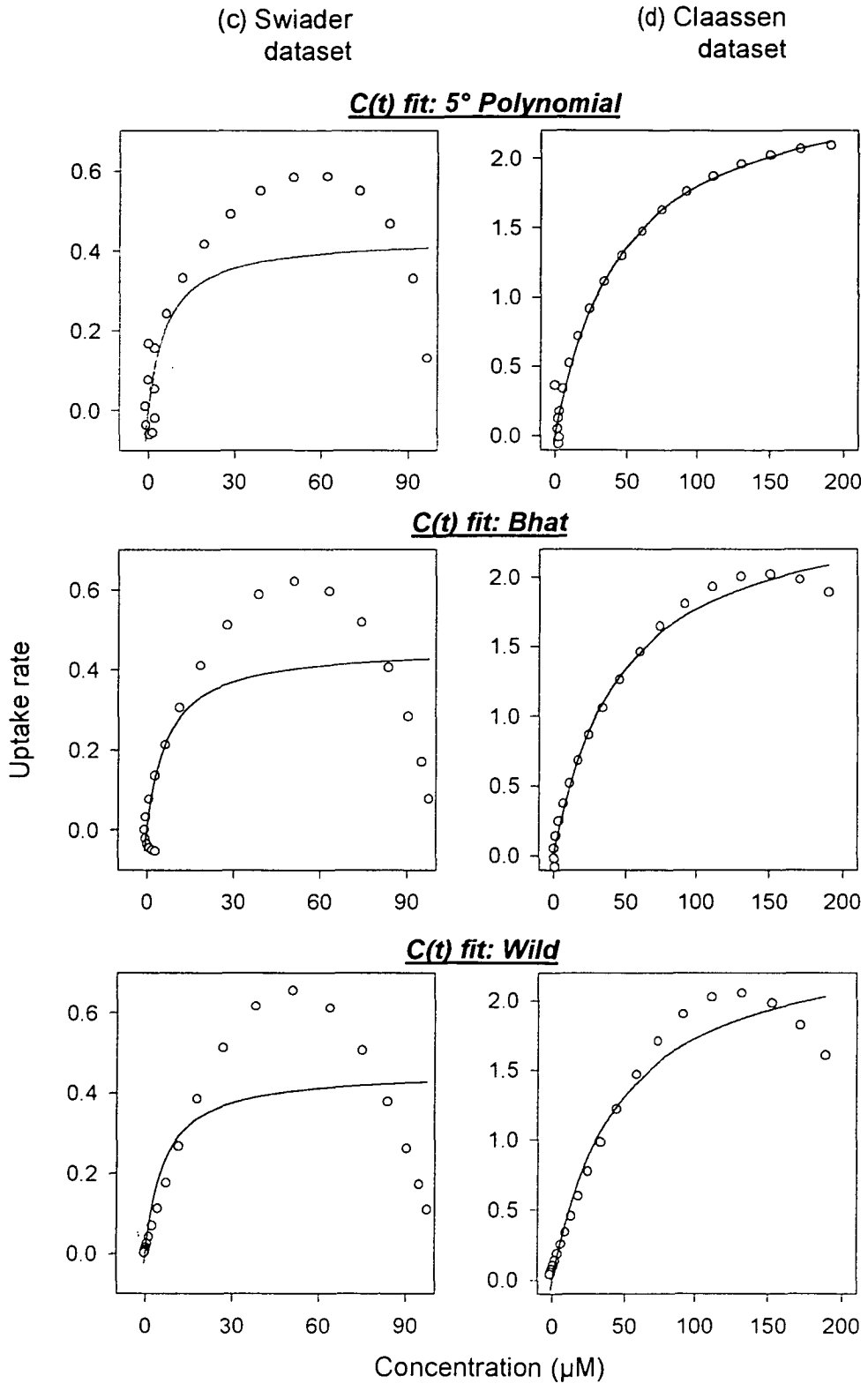


***Figure 3.3. (cont.)*** *The V(C) data produced for four datasets using the three types of C(t) fitting functions: 5° polynomial, Bhat function, and Wild function. The V(C) data are represented as symbols whilst the lines are produced from fitting the standard M-M function to these data. Datasets are as in Figure 3.2.*



### 3.3.2 M-M fits to V(C) data

#### *3.3.2.1 Unmodified datasets*

In only one of the twelve cases (Figure 3.3 (d) 5° polynomial) did the V(C) data produced from fitting various functions to the C(t) data show characteristic saturation kinetics. This lack of saturation kinetics was evident in the poor fit of the simple M-M function (Equation 3.5) to the data from the different curve fits (Figure 3.3). The main cause of the poor fit of the M-M function was the reduced uptake rates at high concentrations. Although the fits of the M-M function were quite poor, the kinetic parameters ($K_m$ and $V_{max}$) estimated for each C(t) function were similar (Table 3.3, unmodified).

The $K^+$ depletion curve of Claassen and Barber (Figure 3.3 d.) is best fitted by the M-M relationship, but even so, at high concentrations only the data produced from the 5° polynomial closely fitted the M-M relationship. The Laine and Swiader data were poorly fitted by the M-M relationship (Figure 3.3, b and d) because the reduction in uptake rates at higher concentrations reduced the $V_{max}$ of the fitted M-M curves.

#### *3.3.2.2 Modified datasets*

There appeared to be two areas of the V(C) curves leading to the poor fits of the standard M-M function and these were the uptake rates at high and low concentrations (Figure 3.3). At low concentrations, once depletion curves had reached $C_{min}$, there were some erroneous uptake rates caused by the curve fitting functions wavering about $C_{min}$. At high concentrations, the V(C) curves showed a reduction in uptake, the extent of which was dependent on the C(t) fitting function used. Assuming the low and high concentration uptake values were artefacts of the fitting process, these points were removed to try and improve the fit of the M-M function. All uptake values occurring after the maximum uptake measurement had been reached were removed. All low concentration values, where either the uptake rate or the concentrations were below zero were also removed. The modified curves were then re-fitted to the M-M function (Figure 3.4).

*Table 3.3. Parameters estimated from fitting the M-M relationship to various V(C) curves which were calculated using the different C(t) fitting functions.*

| | | (a) $V_{max}$ (Relative) | | | | (b) $K_m$ (μM) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Estimated | Unmodified | Modified Dataset | | Unmodified | Modified Dataset | | Using linear |
| Dataset | C(t) fit | from linear regression (raw data) | Dataset (no $C_{min}$) | without $C_{min}$ | with $C_{min}$ | Dataset (no $C_{min}$) | without $C_{min}$ | with $C_{min}$ | regression $V_{max}$ |
| | 5° polynomial | 0.63 | 0.98 | 1.01 | 0.87 | 30.1 | 28.0 | 16.6 | 5.5 |
| **Edwards** | Bhat function | 0.63 | 0.97 | 1.19 | 0.98 | 31.9 | 40.4 | 22.8 | 6.5 |
| | Wild function | 0.63 | 0.91 | 1.98 | 1.44 | 27.7 | 87.7 | 49.1 | 9.4 |
| | 5° polynomial | 0.83 | 0.86 | 1.31 | 1.03 | 35.2 | 55.6 | 23.8 | 11.9 |
| **Laine** | Bhat function | 0.83 | 0.82 | 1.29 | 1.11 | 36.5 | 59.3 | 36.7 | 15.3 |
| | Wild function | 0.83 | 0.77 | 1.95 | 1.65 | 29.1 | 138.9 | 98.4 | 23.4 |
| | 5° polynomial | 0.55 | 0.43 | 0.70 | 0.77 | 6.49 | 11.81 | 18.0 | 4.9 |
| **Swiader** | Bhat function | 0.55 | 0.46 | 0.85 | 0.95 | 7.05 | 11.50 | 26.1 | 5.9 |
| | Wild function | 0.55 | 0.45 | 1.16 | 1.16 | 6.30 | 35.92 | 36.0 | 7.5 |
| | 5° polynomial | 1.72 | 2.64 | 2.59 | 2.61 | 46.8 | 43.9 | 44.7 | 11.6 |
| **Claassen** | Bhat function | 1.72 | 2.60 | 2.77 | 2.88 | 46.9 | 51.6 | 59.1 | 14.6 |
| | Wild function | 1.72 | 2.52 | 3.42 | 3.56 | 46.2 | 79.2 | 87.7 | 17.3 |

***Figure 3.4.*** *Modified V(C) curves and curves resulting from fitting the M-M function to these curves. Symbols represent V(C) values and lines the fitted M-M functions.*

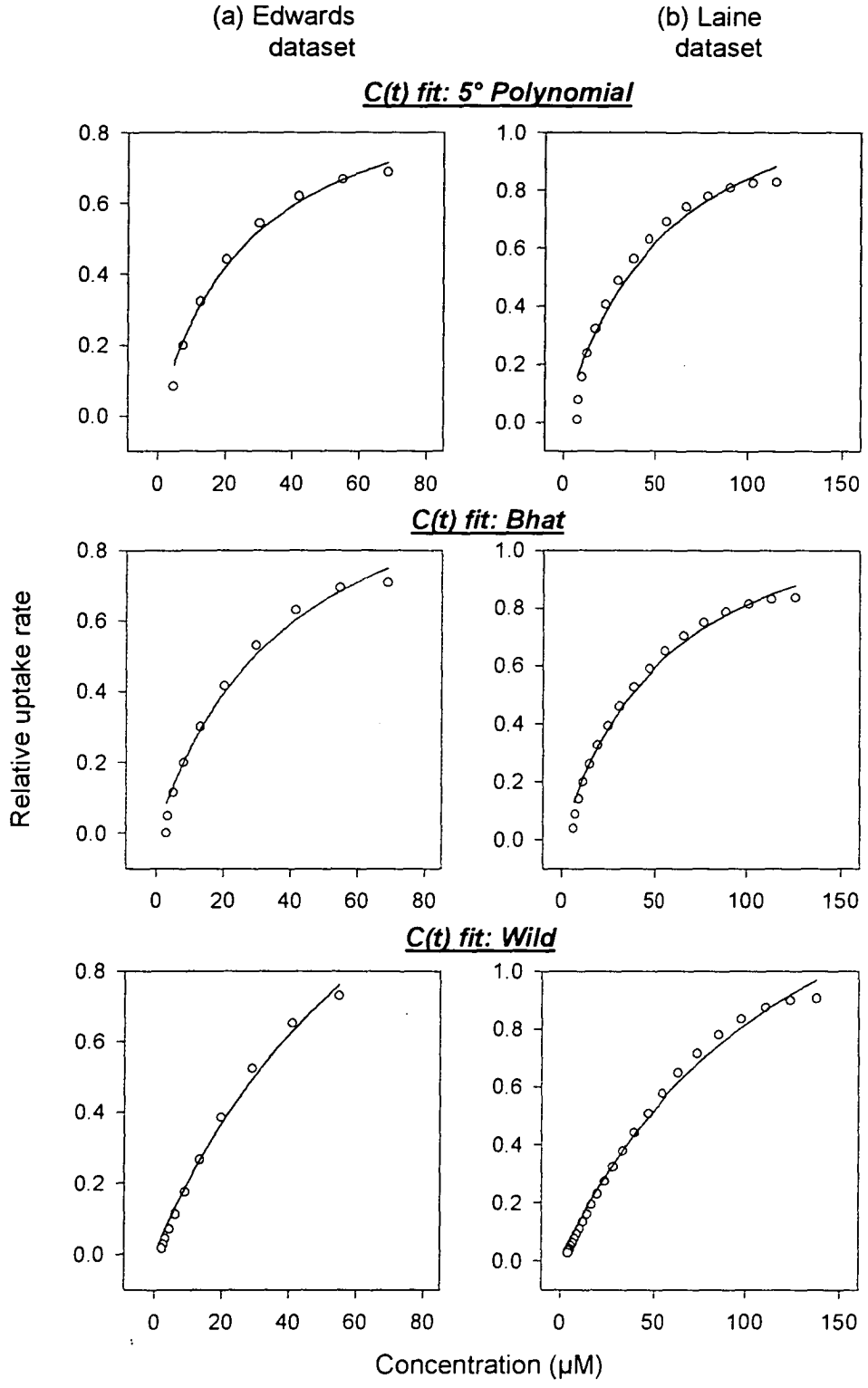
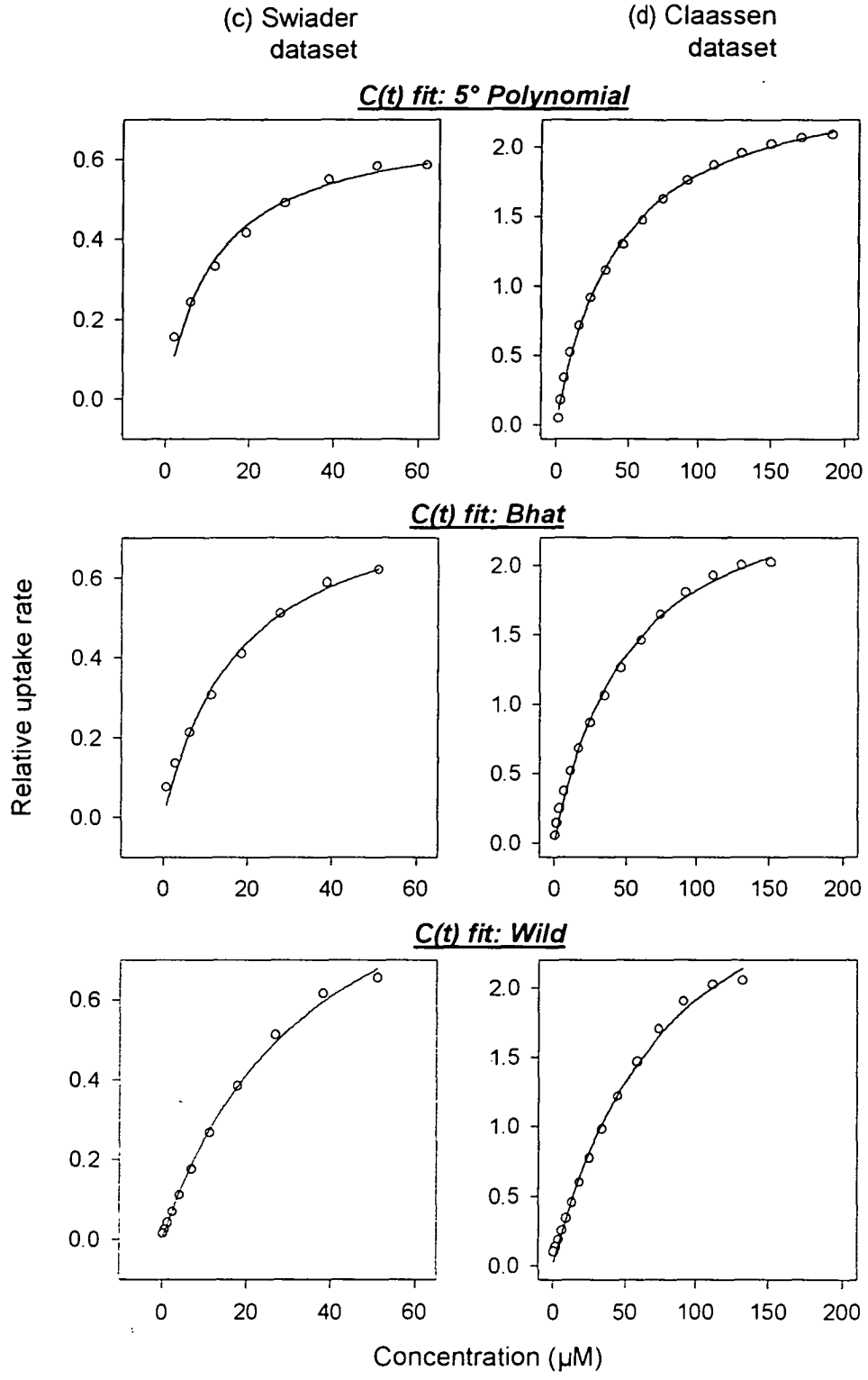


***Figure 3.4 (cont.)*** *Modified V(C) curves and curves resulting from fitting the M-M function to these curves. Symbols represent V(C) values and lines the fitted M-M functions.*



The fit of the M-M function was much better following removal of high and low concentration values. For each dataset and fitting method the M-M function was a much closer fit to the V(C) data than was the case for the unmodified V(C) data. At the highest concentrations the M-M functions did not asymptote in the same way the V(C) data did and this lead to higher parameter estimates (Table 3.3, modified data). The $V_{max}$ estimates for some of the datasets were doubled for the modified data compared with those estimated for the unmodified data. Unlike the unmodified data, where parameters for each fitting function were similar, the parameter estimates for the modified data showed large differences (up to 96% for $V_{max}$ , and up to 313% for $K_m$) between the 3 different C(t) fitting functions.

#### *3.3.2.3 Including $C_{min}$*

The M-M fit to the modified data was much better than to the unmodified data but it still did not accurately fit the data over the full range of the modified concentration data (Figure 3.4). The revised M-M function including $C_{min}$ (Equation 3.1) was fitted to the modified concentration versus uptake data of Figure 3.4.

Fitting the M-M function with $C_{min}$ resulted in an even better fit of the modified V(C) data (Figure 3.5). The values of $K_m$ estimated this way for the Edwards and Laine datasets were less than with the standard M-M function (without $C_{min}$, Table 3.3). The biggest change in $K_m$ estimates were for the Edwards dataset, where for each C(t) fit type the values of $K_m$ were roughly half those estimated from the application of the standard M-M function. The estimated $C_{min}$ values were similar but less ($\approx$ 40%) than the measured $C_{min}$ values (measured $C_{min}$ values were: Edwards, 4 μM; Laine, 10 μM; Swiader, 1μM; Claassen, 3 μM) and did not vary greatly due to C(t) fitting function. Estimates of $V_{max}$ were not greatly different from when the standard M-M function was fitted to these modified datasets.

#### *3.3.2.4 Including linear regression $V_{max}$*

Values of $V_{max}$ estimated as the slopes of the initial part of the raw C(t) data were uniformly less than the $V_{max}$ values estimated using the M-M functions (Table 3.3). $V_{max}$ values estimated from linear regression were incorporated into the revised M-M function as independent variables and fitted to the modified V(C) data.

***Figure 3.5.*** *Modified V(C) curves fitted to the revised M-M function (Equation 3.1). Symbols represent V(C) values and lines the fitted M-M functions.*

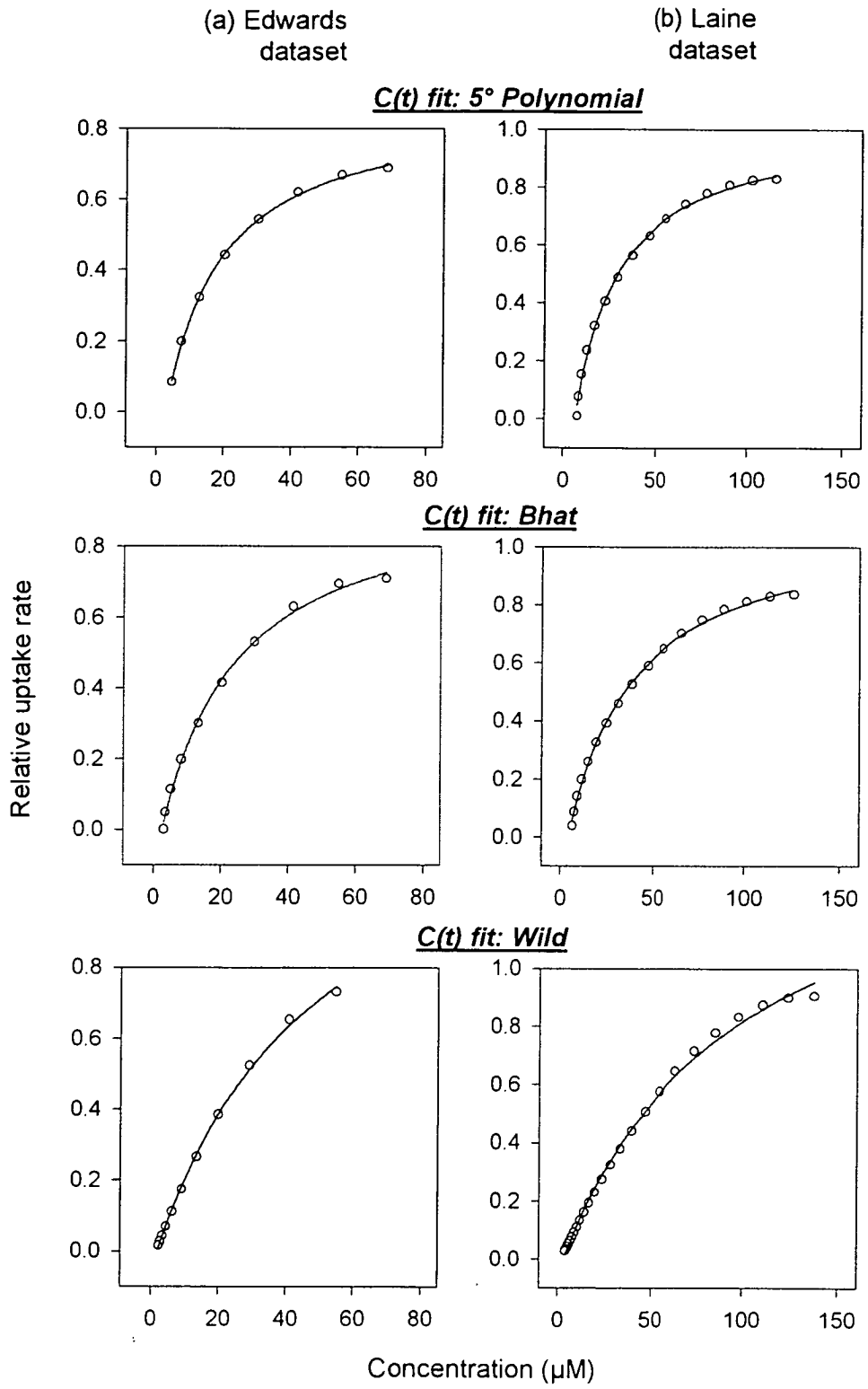


***Figure 3.5. (cont.)*** *Modified V(C) curves fitted to the revised M-M function (Equation 3.1). Symbols represent V(C) values and lines the fitted M-M functions.*

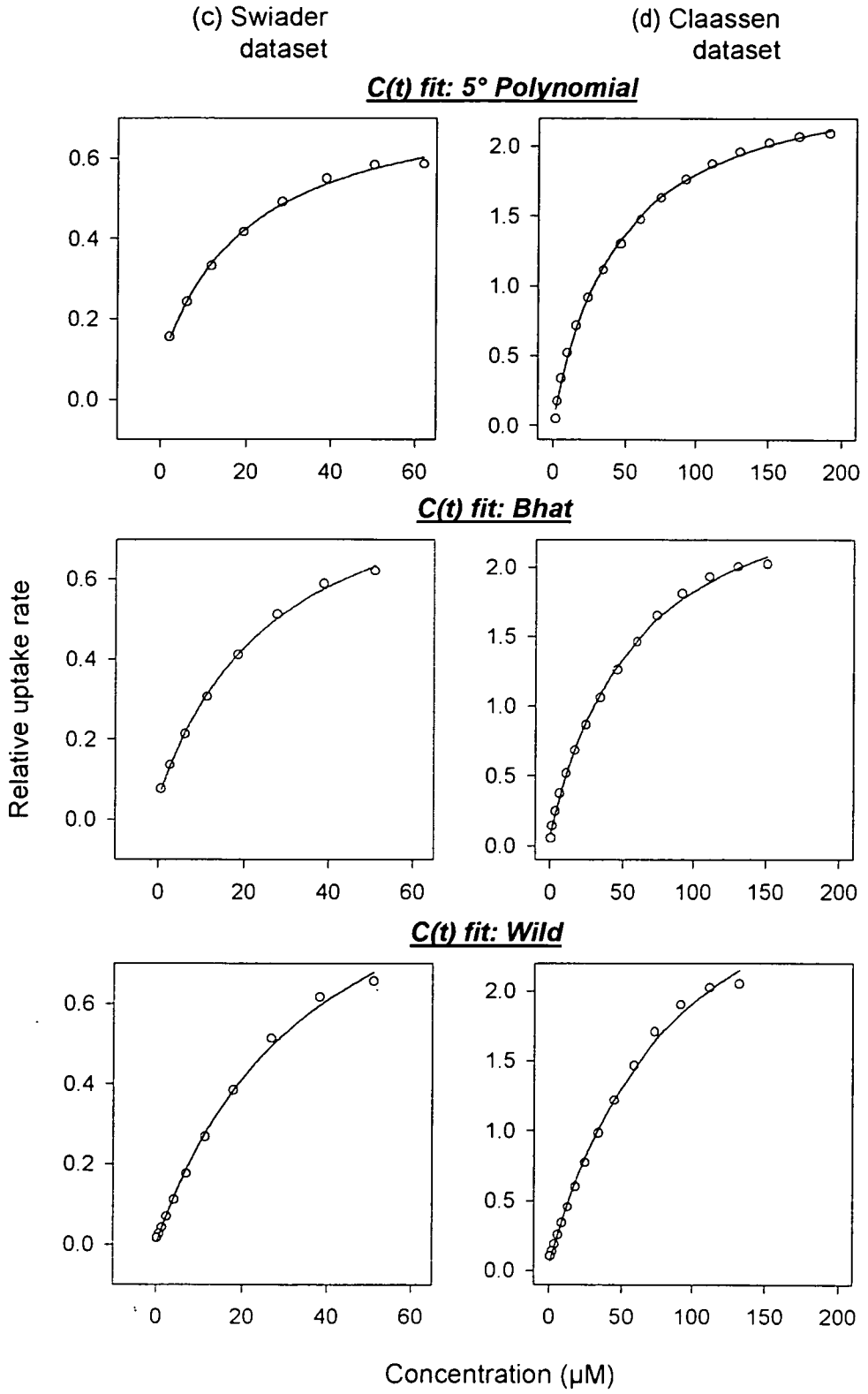


***Figure 3.6.*** *Modified V(C) curves fitted to the revised M-M function (Equation 3.1) including $V_{max}$ values estimated from linear regression as independent variables. Symbols represent V(C) values, and lines the fitted M-M functions.*

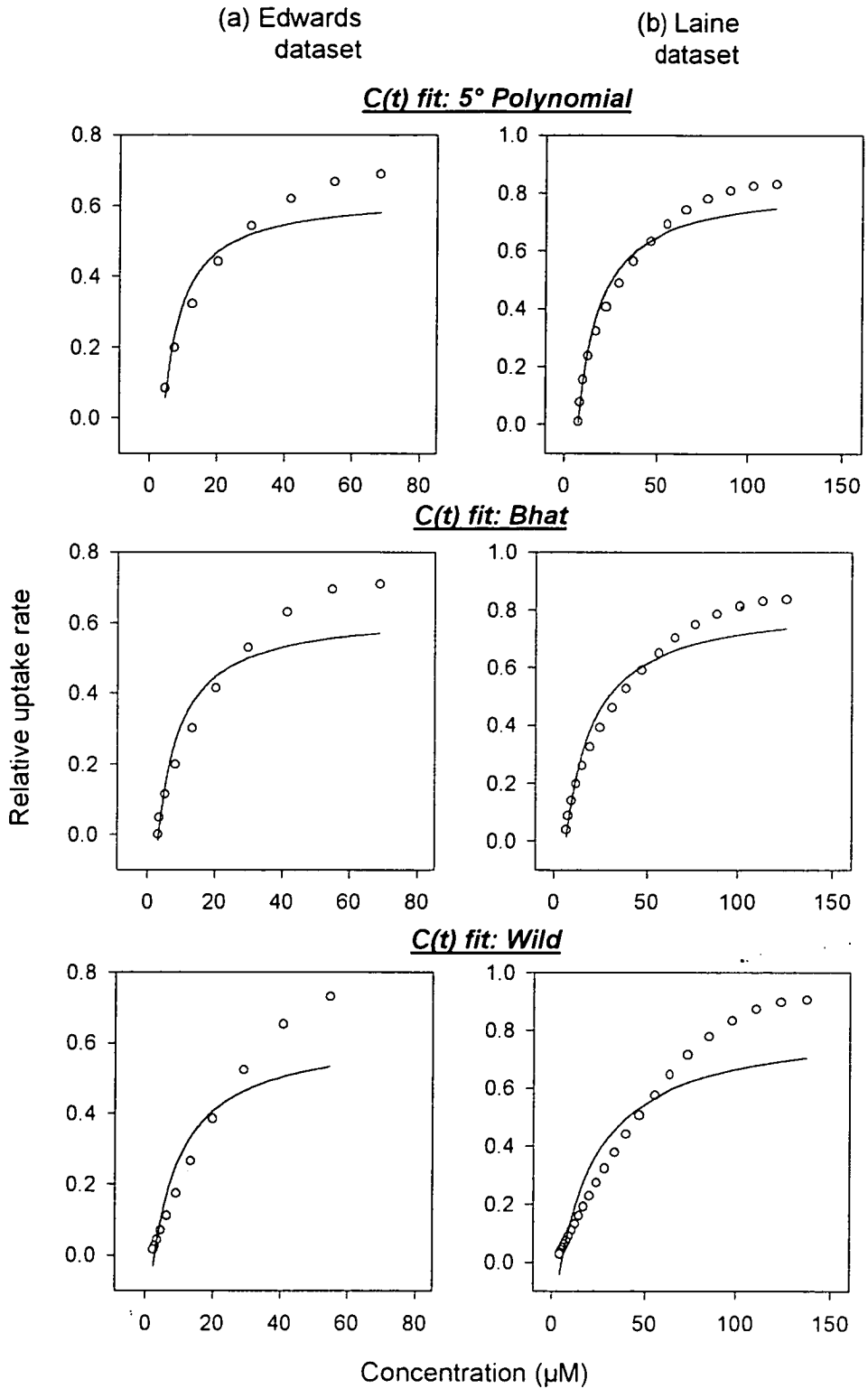
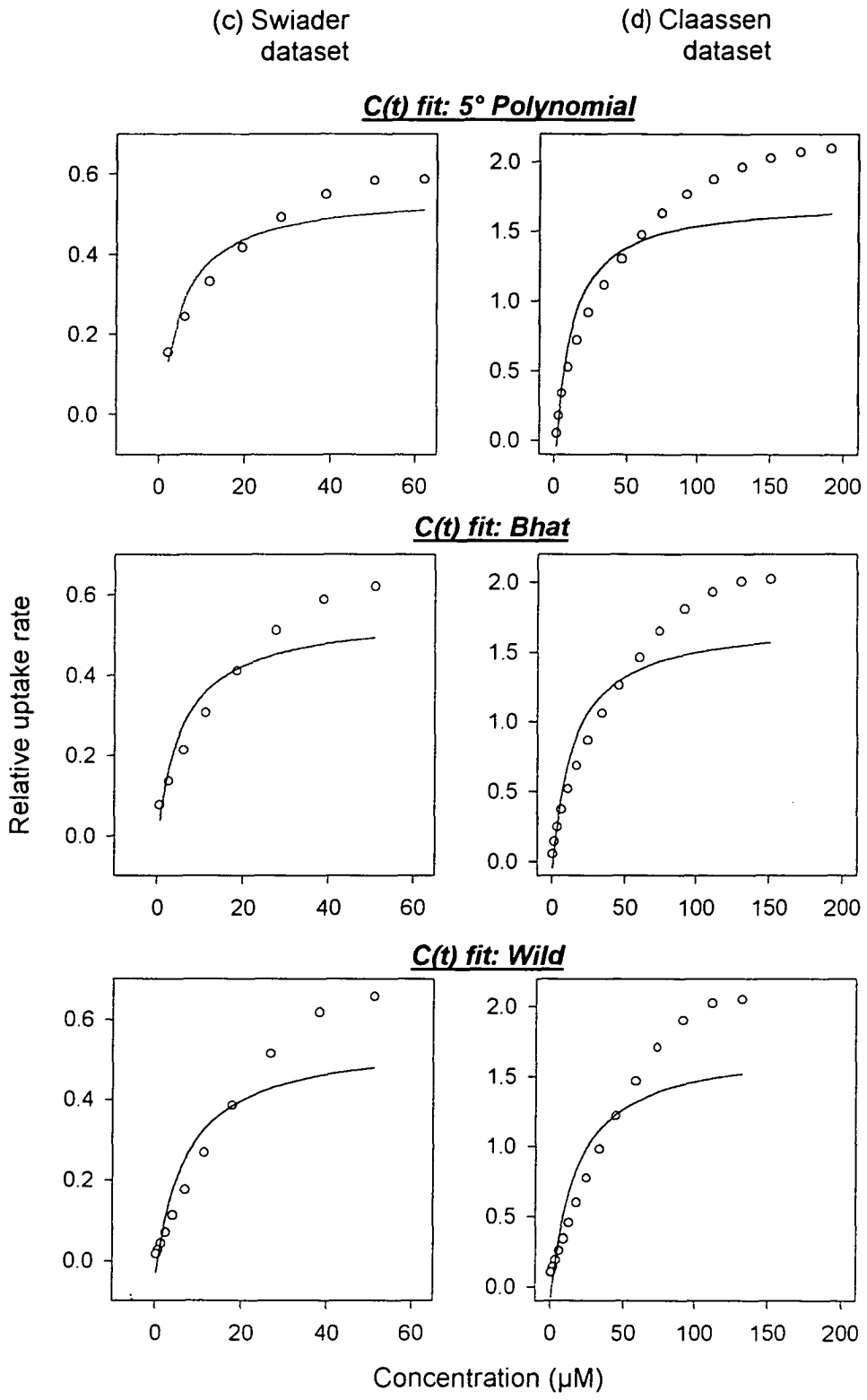


***Figure 3.6. (cont.)*** *Modified V(C) curves fitted to the revised M-M function (Equation 3.1) including $V_{max}$ values estimated from linear regression as independent variables. Symbols represent V(C) values, and lines the fitted M-M functions.*



The fit of the M-M function, including $V_{max}$ values estimated from linear regression, gave very poor fits to the V(C) data (Figure 3.6). None of the datasets, regardless of C(t) function used, were accurately fitted by the M-M function. The poorness of fit was similar to that of the simple M-M function to the unmodified V(C) data (Figure 3.3).

## *3.4 Discussion*

### 3.4.1 C(t) fitting functions

Based on visual inspection, the three C(t) functions (5° polynomial, Bhat, and Wild) closely fitted the C(t) data and appeared suitable for use in describing C(t) curves (Figure 3.2). The V(C) curves produced from these fits show however that the slight differences in curve fitting lead to large differences in uptake rates calculated from the differentiated functions in some parts of the concentration ranges (Figure 3.3). The unmodified dataset that was closest in behaviour to the M-M relationship was the Claassen $K^+$ dataset (Figure 3.2 (d)). Of the three C(t) fitting functions, that which produced the best fit to the Claassen dataset was the 5° polynomial (Figure 3.3 (d), 3.4 (d)). For the modified datasets there was consistent overestimation by the M-M functions at the highest concentrations for all the C(t) fitting functions (Figure 3.4), but the least overestimation was shown with the 5° polynomial method. Based on these observations the 5° polynomial was the best of the three C(t) fitting functions.

### 3.4.2 Fitting M-M functions to V(C) data

For each of the 4 unmodified datasets, the simple M-M function yielded similar parameters for both $V_{max}$ and $K_m$ for all three C(t) fitting functions (Figure 3.3, Table 3.3). However, apart from the 5° polynomial fit to the Claassen dataset, none of the fitted M-M functions actually fitted the V(C) data. The parameter estimates obtained in this way seem of little relevance as they do not describe the V(C) relationship.

The modification of the V(C) curves, to remove the high and low concentration points on the basis that they are experimental artefacts, greatly improved the fit of the simple M-M function (Figure 3.4). The inclusion of a $C_{min}$ term to the M-M function even further improved the fit of the M-M function (Figure 3.5). The parameters estimated from these curves were probably the best estimates possible with these

modified data. However, there were large differences in parameters estimated for the V(C) data produced from the three C(t) fitting functions (Table 3.3). The $K_m$ parameters estimated for the 5° polynomial-produced data were generally half of those estimated for the Wild-produced data, and in the case of the Laine dataset were only a quarter of the value. The differences between $K_m$ values estimated from the 5° polynomial data and the Bhat-produced data were smaller, but for the 5° polynomial data were consistently less. Based on there being less systematic deviation between the 5° polynomial data and the M-M functions, the $K_m$ estimates from the 5° polynomial data are assumed to be more reliable.

The relative differences between the $V_{max}$ estimates from the different C(t) functions were much less than between the estimates of $K_m$ (Table 3.3). Once again, due to the better fit of the revised M-M function to the 5° polynomial-produced data, the $V_{max}$ estimates from the 5° polynomial-produced data are thought to be the most accurate.

The values of $V_{max}$ estimated by fitting the M-M functions (Equations 3.1, 3.5) were in all cases higher than the $V_{max}$ found by linear regression of the initial part of the depletion curve (Table 3.3). The value of $V_{max}$ estimated from linear regression was the uptake rate before any noticeable change in uptake occurred. The $r^2$ for the regression lines used was in all cases greater than 0.995. In this fitting it was assumed that there was no change in the uptake rate over the concentrations used. This assumption is not justified by any of the C(t) fitting functions and according to the M-M relationship the increase in uptake rates slows, but is not stopped, as concentrations increase. Hence, the $V_{max}$ estimated from linear regression will be underestimated. This difference is evident in the poor fit of the M-M relationship including the linear regression estimates of $V_{max}$ as independent variables (Figure 3.6).

The values of $V_{max}$ estimated from fitting the revised M-M equation to the modified V(C) data were all higher than the highest uptake rate in the V(C) data. The extent of this difference was dependent on the C(t) function used (25% for 5° polynomial, 45% for Bhat, 85% for Wild). This difference occurred because there were insufficient V(C) values at high concentrations. If there were more high concentration values in the region where uptake rates asymptote then this difference would be less. However, in most cases there were higher concentration uptake values

that had been removed because they did not fit the M-M relationship. In view of these differences between the estimates of $V_{max}$ it may be that the best estimate of $V_{max}$ was the highest uptake rate apparent in the V(C) data.

### 3.4.3 Nitrogen in depletion experiments

The main problem with fitting the M-M functions to the V(C) data lay in the reduced uptake rates at high concentrations, which was evident in all three N datasets (Edwards, Laine, Swiader). For the Edwards V(C) data the reduction was slight and it's extent was dependent on the C(t) fitting function (Figure 3.3 (a)), but for the Laine and Swiader datasets the reduction in uptake rate at high concentrations was not simply due to the C(t) fitting function, and was easily apparent in the raw C(t) data. For the Laine dataset, the initial slow rates were thought to be due to efflux caused by pre-treatment in a higher concentration solution than the initial depletion concentration (Laine et al., 1993). However, the Swiader dataset was obtained with plants that had been acclimatised overnight in the same concentration as that of the initial depletion concentration (Swiader and Freiji, 1996), and these also showed an initial slow uptake rate. If it were possible to minimise the initial reduction in uptake rates via manipulation of the pre-treatments then this would aide the parameter estimation process.

Regardless of the cause of the initial reduced uptake rates, the removal of these points from the V(C) data was necessary to adequately fit the M-M function to the data. Unfortunately, removal of these points led to inaccuracies in estimating $V_{max}$. One way to minimise this problem might be to have a higher starting concentration for the depletion. In this way, even after the discarding of any reduced uptake points, enough higher concentration data might be left to enable more accurate estimates of $V_{max}$. The disadvantage of this approach would be that the depletion experiments would last even longer, which could increase the likelihood of changes in uptake properties during the measurement. The Laine and Swiader datasets were for experiments that were almost 10 hours long. Based on the turnover times for nitrate transporters being as low as 4 hours (Clarkson, 1986), it is possible that significant changes in N uptake transport characteristics could be occurring during depletion experiments.

The above results show that the best fit of the C(t) curves can be obtained using a 5° polynomial. The V(C) curves could then be fitted to the revised M-M function including $C_{min}$ as a dependent variable. This process will be straightforward for datasets such as Claassen's. Unfortunately the N datasets used here did not show the same relationship as the Claassen $K^+$ dataset, i.e. they exhibited reduced uptake at high concentration. If possible it would be beneficial to adapt methodologies to minimise the reduced uptake at high concentrations for N datasets. If the reduced uptake rates cannot be experimentally rectified then V(C) datasets must be modified as was done above.

The advantages of directly fitting the M-M function to the V(C) data, as opposed to the Lineweaver-Burk style methods, is that it is simple to assess the fit of the function to the data and that biases due to transformations are avoided (Ritchie and Pravan, 1996).

The cut off point for exclusion of data to produce modified datasets has a big influence on the magnitude of the parameter estimates. The removal of datapoints is subjective, leading to possible problems in terms of comparability of results between different experiments. The removal of points must be completed to some consistent strategy such as was used here, where points greater than the maximum uptake rate, and any negative uptake rates or concentrations were removed. Any removal of points must be acknowledged otherwise there will be no compatibility with other results. Although removal of datapoints is unwanted because of the loss of objectivity, it is far preferable than to estimate parameters regardless of fit to the M-M relationship, such as was the case in Figure 3.3.

That efflux could cause a reduction in (net) uptake at higher concentrations could means that even the highest rate of uptake measured includes some efflux component. According to the suggestion of Claassen and Barber (1974), the most appropriate means of accurately dealing with this problem would be to have some measure of the concentration dependence of efflux that could be incorporated into the M-M relationship. Claassen and Barber (1974) incorporated an efflux term in a similar way but this was constant with respect to concentration and performed a similar role to that of $C_{min}$ in the revised M-M equation (Equation 3.1). However, the problem with this proposition is that efflux would need to be quantified for each experiment,

which in turn would be quite difficult and negate the simplicity of the depletion technique.

## *3.5 Conclusions*

If the C(t) data are very close to the ideal M-M relationship, such as Claassen and Barber (1974), then any fitting function will lead to satisfactory estimates of kinetic parameters. Unfortunately N depletion data did not appear to follow the ideal M-M relationship for the full range of depletion concentrations. V(C) data produced from C(t) data must be modified to fit the M-M function and this process led to large differences between the kinetics parameters estimated using the different C(t) fitting functions. The most reliable function to fit to C(t) data was a 5° polynomial. The most reliable function to fit to V(C) data was the revised M-M relationship which included $C_{min}$ as a dependent variable. It seems likely that much of the variation between N kinetic parameters estimated in different depletion experiments in the literature is due to differences in the data analysis and parameter estimation methods.

# 4. Ammonium and nitrate uptake by roots of *E. nitens*

## *4.1 Introduction*

As previously outlined in Chapter 3, the depletion method is a simple method for measuring N uptake rates by plant roots from solution. Using this method, a number of variables could be tested for their effects on $NH_4^+$ and $NO_3^-$ uptake by *E. nitens*. Several environmental variables have potentially large effects on N uptake characteristics of plant roots, in particular N form, soil pH, temperature, plant N status and growth rate. Because these variables each exhibit a large range of values in eucalypt plantations in south-eastern Australia, any characterisation of N uptake by *E. nitens* roots would be incomplete without consideration of these effects.

### 4.1.1 Inhibition of nitrate uptake by ammonium

There are many cases in the literature where there is an apparent inhibition of $NO_3^-$ uptake by the presence of $NH_4^+$ (Lycklama, 1963; Rao and Rains, 1976; MacKown et al., 1982; Breteler and Siegerist, 1984, Glass et al., 1985; Lee and Drew, 1989; Wieneke, 1992; Ayling, 1993; Kamminga-van Wijk and Prins, 1993; Aslam et al., 1994). There are also cases in the literature were the presence of $NH_4^+$ had no effect on $NO_3^-$ uptake (Neyra and Hageman, 1975; Thibaud and Grignon, 1981; Breteler and Siegerist, 1984; McClure et al., 1990), and does not appear to affect the constitutive $NO_3^-$ transport system (uninduced) (Breteler and Siegerist, 1984). The effect of $NH_4^+$ on $NO_3^-$ uptake can differ between cultivars of the same species. For example, Bloom and Finazzo (1986) found that for two different cultivars of barley, the presence of $NH_4^+$, stimulated $NO_3^-$ uptake in one cultivar, but in another cultivar, $NO_3^-$ uptake was inhibited by 50%. Effects of $NH_4^+$ on $NO_3^-$ uptake were reduced when barley plants were exposed to $NH_4^+$ during growth (Lee and Drew, 1989).

There has been debate as to whether the effect of $NH_4^+$ on $NO_3^-$ uptake is via an inhibition of $NO_3^-$ influx, or a stimulation of $NO_3^-$ efflux (Deane-Drummond and Glass, 1983; Lee and Drew, 1989; Aslam et al., 1994). The results of studies where membrane potential was measured show a rapid depolarisation upon the addition of $NH_4^+$, and it may be this depolarisation that causes short term inhibition of $NO_3^-$ influx

(Ullrich et al., 1984; Ayling, 1993; Meharg and Blatt, 1995). It has also been proposed that the inhibition is due to feedback effects of $NH_4^+$ metabolites (King et al., 1993). These two different lines of evidence may have resulted from examining two sides of the process, one looking at immediate effects of $NH_4^+$ on the membrane potential, and a longer term effect due to uptake regulation and assimilation, perhaps affecting efflux.

Inhibition of $NO_3^-$ uptake by $NH_4^+$ is effective over a wide range of $NH_4^+$ concentrations from the low μM (1 μM) range to the high mM (50mM) range (Lee and Drew, 1989; Kamminga-Van Wijk and Prins, 1993). In eucalypt plantation soils $NH_4^+$ and $NO_3^-$ concentrations after fertilisation commonly reach >1 mM levels that remain for long periods (> 6 months) (Hingston and Jones, 1985; Smethurst et al., 1997). An inhibition of $NO_3^-$ uptake by $NH_4^+$ could have important implications for fertiliser management practises.

### 4.1.2 pH

The pH of forest soils in south-eastern Australia is generally between pH 6 and pH 4 (Judd et al., 1996; Cromer, pers. comm.), which is more acidic than most agricultural soils. Soil pH affects several aspects of the nutrient supply characteristics of soils. In particular, low soil pH can reduce nitrification rates, which in turn leads to high ratios of $NH_4^+$: $NO_3^-$ (Haynes and Goh, 1978).

There are two main facets to the relationship between pH and $NH_4^+$ and $NO_3^-$ uptake, firstly, the effect that $NH_4^+$ and $NO_3^-$ nutrition have on the root environment, and secondly, the effect that pH has on $NH_4^+$ and $NO_3^-$ uptake. Ammonium uptake generally results in the acidification of the growth medium, whilst $NO_3^-$ uptake generally results, to a lesser extent, in the alkalisation of the growth medium (Haynes and Goh, 1978). One of the reasons why a combined nitrogen source can yield high growth rates is that the combination of $NH_4^+$ and $NO_3^-$ uptake leads to a relatively stable pH compared with that of the single N sources (Hewitt, 1966). The cause of the pH changes due to $NH_4^+$ and $NO_3^-$ nutrition is that $NH_4^+$ assimilation produces at least one proton per $NH_4^+$ assimilated, and $NO_3^-$ assimilation leads to the production of almost one $OH^-$ per $NO_3^-$ assimilated (Raven and Smith, 1976).

The effect of solution pH on uptake of $NH_4^+$ and $NO_3^-$ is unclear, as the response found in different plants and under different conditions varies widely. There have been occurrences where uptake of $NH_4^+$ has increased with increasing pH (Fried et al., 1965; Carrodus, 1969; Munn and Jackson, 1978; Marcus-Wyner, 1983; Vessey et al., 1990), but there have also been a number of circumstances where $NH_4^+$ uptake has increased with decreasing pH (Wang et al., 1993b; McFarlane and Smith, 1982).

For $NO_3^-$ uptake, as with $NH_4^+$ uptake, the effect of pH is variable. In a number of studies, $NO_3^-$ uptake increased with decreasing pH (Rao and Rains, 1976; Munn and Jackson, 1978; Marcus-Wyner, 1983; Vessey et al., 1990; McClure et al., 1990), but in at least one study, uptake increased with increasing pH (Doddema and Telkamp, 1979). Still further studies have found that there was a pH optimum for $NO_3^-$ uptake, with pH more alkaline or acidic than the optimum value leading to reduced uptake (Lycklama, 1963; Peuke and Tischner, 1991; Aslam et al., 1995). Aslam et al., (1995) found that the decrease in uptake at acidic pH was due to a stimulation of efflux, whilst at alkaline pH, the reduction in uptake was due to a reduction in influx.

Most experiments to determine pH effects on uptake were only at one N concentration. It has been proposed that pH may directly affect the $NO_3^-$ transporter leading to a change in the $K_m$ for $NO_3^-$ uptake, but not the $V_{max}$ (Meharg and Blatt, 1995). These authors suggest that this difference may help explain the different effects of pH on $NO_3^-$ uptake reported. It is possible that a similar system of pH effects could occur for $NH_4^+$ uptake, although at least for one plant, the unicellular alga *Chlorella*, the $K_m$ for $NH_4^+$ did not change between pH 4 and pH 6 (Schlee and Komor, 1986).

### 4.1.3 Temperature

*E. nitens* plantations in Tasmania are generally between 500-700 meters elevation. In these regions temperatures are generally cold with average winter air temperatures ranging between 4 and 7 °C (Davies, 1965). These low temperatures will affect rates of both plant growth and N uptake.

Low temperatures lead to reduced uptake rates of both $NH_4^+$ and $NO_3^-$. There appears to be two ways in which temperature affects the actual uptake process. Firstly, decreased activity of the $H^+$-ATPase means there is less energy for secondary transport. Secondly, changing the fluidity of the plasma membrane directly affects the

activity of transporters (Kennedy & Gonsalves, 1988). Effects of temperature on biochemical processes are generally referred to in terms of the temperature coefficient ($Q_{10}$) which is the difference in rate associated with a temperature change of 10°C (Nobel, 1983). For $NH_4^+$ influx in rice roots, $Q_{10}$ values ranged from 1.2-2.6 (Wang et al., 1993b), whilst for $NO_3^-$ influx in barley, $Q_{10}$ values ranged between 1.2-3.8 (Glass et al., 1990). The trend seems to be that $Q_{10}$ values tend to be lower for the HATS than the LATS, and they also appear to be higher between low temperatures (5-10°C) than between high temperatures (10-20°C, 20-30°C). The higher values in the low temperature range may relate to transition temperatures, possibly related to a phase change in the plasma membrane, found for $NH_4^+$ and $NO_3^-$ uptake in *Lolium* above or below which $Q_{10}$ values were similar (Clarkson and Warner, 1979).

For some plants the $NH_4^+$ and $NO_3^-$ uptake processes are affected differently by temperature. For example, the preference for $NH_4^+$ over $NO_3^-$ was increased at low temperatures in perennial ryegrass (Lyklama, 1963; Clarkson and Warner, 1979; Macduff and Hopper, 1986, Clarkson et al., 1986; Macduff and Wild, 1989), possibly due to increased sensitivity of $NO_3^-$ uptake to inhibition by $NH_4^+$ (Deane-Drummond and Glass, 1983, Clarkson et al., 1992). However, this effect may not be universal as preference for $NH_4^+$ remained unchanged with reduced root temperatures in oilseed rape (Macduff et al., 1987).

Temperature also affects plant growth. Exposing roots to low temperatures leads to reduced root growth and eventually reduced growth of the whole plant (Clarkson et al., 1986; Macduff et al., 1987; Clarkson et al., 1992; Macduff et al., 1994). These growth effects change the demand for nutrients and can make it difficult to interpret direct effects of temperature on uptake processes. Roots grown at low temperatures can exhibit an increased uptake capacity when subsequently exposed to higher temperatures, which may be a response of the root to meet increased shoot demands (Clarkson and Warner, 1979; Deane-Drummond and Glass, 1983; Macduff et al., 1994; Bigot and Boucard, 1994).

Uptake experiments, including those described in this chapter, are generally carried out with both roots and shoots kept at the same, generally ambient, temperatures ranging from 20-25°C. This situation may not be very realistic, as it is very rare for root and shoot temperatures to coincide (Rorison et al., 1983), and in

many parts of temperate Australia, to be this high. To increase the universality of an N uptake characterisation it is therefore important to quantify the effects of temperature differentials between roots and shoots on N uptake.

### 4.1.4 RGR and Nutrient Status

Plant responses to low nutrient supply can involve either or both an increase in root size with respect to the shoot (Ingestad and Lund, 1986) and an increase in the uptake capacity of the roots by either an increase in $V_{max}$ or a decrease in $K_m$ (Gutschick, 1993). The extent to which these two aspects are invoked is uncertain. The $V_{max}$ for $NO_3^-$ uptake by barley was found to increase from low to medium RGR but then decrease at higher RGR (Mattsson et al., 1991). A similar result was seen for both pea and duckweed (Oscarson et al., 1989). The $K_m$ for nitrate uptake by pea and duckweed was unaffected by RGR (Oscarson et al., 1989), whilst for barley $K_m$ increased with increasing RGR (Mattsson et al., 1991).

For most experiments in this chapter, plants were grown in bulk solution culture, the N concentration of which was 4 mM (1.6 mM $NH_4^+$, 2.5 mM $NO_3^-$). Plants showed good growth at these concentrations, but these concentrations are in the maximum range of concentrations found in the field (Smethurst, pers. comm.). The soil solution N concentrations in a well fertilised plantation might reach these mM levels, but it would be very unlikely to find these concentrations in undisturbed native forest soils. The high concentrations used in basic solution culture techniques were designed to minimise concentration variations between solution changes (Asher and Edwards, 1983), but they might not be representative of conditions commonly experienced by plants in the field.

An alternative solution culture method, the relative addition rate (RAR) method (also termed nutrient flux density approach), allows plants to be grown under highly controlled degrees of nutrient limitation (Ingestad and Ågren, 1992). In the RAR method, a limiting nutrient is supplied at an exponentially increasing rate that sets the relative growth rate of the plant(s) whilst maintaining a constant concentration of the nutrient in plant tissues (Marschner, 1995). This method avoids many of the artefacts of bulk culture systems and can be a reliable method of producing plants of contrasting internal nutrient status and demand (Ingestad and Lund, 1986).

No studies of this nature have been reported for eucalypts, therefore, in characterising N uptake kinetics of *E. nitens* it was necessary to evaluate responses to internal N status and growth rate.

### 4.1.5 Objectives

The objectives of research described in this chapter were to answer the following questions:

- Are $NH_4^+$ or $NO_3^-$ uptake rates sensitive to the presence of the alternative source of N?
- How sensitive are $NH_4^+$ and $NO_3^-$ uptake rates to pH changes in the range 4 to 6? Do eucalypts show any evidence of adaptation to low pH?
- How sensitive are $NH_4^+$ and $NO_3^-$ uptake rates to changes in temperature of the culture solution between 10°C and 20°C? Do roots have greater uptake capacity when cold acclimatised? What is the timescale of any acclimation of uptake rates to changes in temperature?
- To what extent does nutrient status or RGR affect $NH_4^+$ and $NO_3^-$ uptake rates by *E. nitens*? How does *E. nitens* adapt to growth-limiting N levels?
- What are the relative uptake rates of $NH_4^+$ and $NO_3^-$ by *E. nitens* under a variety of pH, temperature, N status and growth rate combinations?

## *4.2 Methods*

### 4.2.1 Plant culture

The seeds used in these experiments were *E. nitens* supplied by North Forest Products (North Eucalypt Technologies). The seeds were selected from a single open pollinated tree in a seed orchard (Macalister provenance, seedlot 102).

The composition of the nutrient solution was based on those developed for *Betula* by Ingestad and Lund (1986). This nutrient solution was selected instead of more commonly used recipes because *E. nitens*, also being a cold-climate tree species, should have similar nutrient requirements to birch. The chosen nutrient solution had also been successfully used for *E. grandis* (Cromer and Jarvis, 1990). The

composition of the nutrient solution is outlined in Table 4.1. The final concentrations of $NH_4^+$ and $NO_3^-$ in the solution were 1.6 and 2.5 mM, respectively.

*Table. 4.1. Nutrient concentrations in growth solutions (based on Ingestad and Lund, 1986). There were two stock solutions used (A and B) which for bulk solution culture were diluted to concentrations shown.*

| Stock | Nutrient | Stock conc. (M) | Dil. conc. (mM) | Stock | Nutrient | Stock conc. (mM) | Dil. conc. (μM) |
|---|---|---|---|---|---|---|---|
| A | $NH_4NO_3$ | 2.80 | 1.60 | B | $HNO_3$ | 50 | 28.57 |
| A | $KNO_3$ | 0.41 | 0.24 | B | $MnSO_4.H_2O$ | 7.28 | 4.16 |
| A | $KH_2PO_4$ | 0.10 | 0.06 | B | $H_3BO_3$ | 18.5 | 10.57 |
| A | $K_2SO_4$ | 0.26 | 0.15 | B | $CuCl_2.2H_2O$ | 0.47 | 0.27 |
| A | $K_2HPO_4$ | 0.32 | 0.18 | B | $ZnSO_4.7H_20$ | 0.46 | 0.26 |
| B | $Ca(NO_3)_2.4H_2O$ | 0.17 | 0.10 | B | $Na_2MoO_4.2H_20$ | 0.07 | 0.04 |
| B | $Mg(NO_3)_2.6H_2O$ | 0.35 | 0.20 | B | FeEDTA(Na) | 87.5 | 50.00 |

Seeds were surface sterilised by soaking in NaClO (1% Cl) for 15 minutes. After sterilising, seeds were thoroughly rinsed and then planted into washed river sand and kept moist with de-ionised (DI) $H_2O$, in the dark, at 20°C. Plants were left in sand until the second set of true leaves were fully expanded (approximately 1 month). Two weeks after sterilisation, dilute nutrient solution was added to the containers, and after three weeks the plants were taken to the glasshouse for extra light. When transferred to solution culture (after 4 weeks) water was used to carefully remove sand from roots and then seedlings were inserted into foam collars. The collars were made from disks of black open cell foam rubber. The disks were 20 mm in diameter with a single cut on one side into the centre. The stem of the seedling was inserted into the centre of the disk such that the root:shoot junction was clear below the foam. When in the culture unit this collar successfully excluded light from above.

The culture units consisted of 35 litre rectangular tubs (600 mm × 400 mm × 220 mm) with a lid (800 mm × 600 mm) made from grey 5 mm thick PVC sheet. Thirty-five 17 mm holes were evenly spaced in the lid within which plants in foam collars were placed. Polystyrene foam sheeting (30 mm thick) was placed around the sides of the tubs for insulation. Once assembled, the culture units excluded light from the roots and culture medium.

A PVC cooling coil was placed within the culture tubs. Tap water (10°C) was circulated through the cooling coil and it's flow regulated such that the temperature (unless otherwise specified) was kept at 20 ± 2 °C. Aquarium pumps supplied air to perforated acrylic tubing at the base of the culture tubs to aerate the solution. Every second day, pH, conductivity and temperature of the solution was recorded. The pH was adjusted to approximately pH 4.2 with KOH and $H_2SO_4$. Solutions were changed twice weekly.

Plants were grown in a temperature controlled glasshouse with the day temperature maintained at 20°C and night at 14°C. Natural light was supplemented by 400 W metal halide lamps positioned above each culture unit. The average PPFD (photosynthetic photon flux density) was 600 μmol $m^{-2}$ $sec^{-1}$ at the leaf surface. Due to the supplemental light, temperatures in the region of the shoots were greater than the glasshouse temperature and averaged 25°C. Day length was set to 14 hours.

**Nitrogen source and pH experiments**

To investigate the effects of N source and pH on N uptake, plants were grown as described above for 7 weeks.

**Temperature experiment**

To investigate temperature effects on N uptake, plants were grown for five weeks as for the pH experiments, then half of the plants of one culture were removed to another culture unit. The cooling flow in the original unit was increased and solution temperature maintained at 11°C (± 2°C). The cooling flow in the second unit was reduced and the temperature maintained at approximately 21°C (± 2°C). Plants were kept under these conditions for two weeks before measurement.

**Relative addition rate and nutritional status experiment**

The RAR approach (Ingestad and Lund, 1986) involves supplying nutrients at short intervals (hourly in the units used) at exponentially increasing rates based on the expected nutrient requirement of the plants. As plant size and their nutrient requirements increase so does the rate of nutrient supply. A limiting nutrient can be supplied at high to low exponentially increasing rates leading to a range of constant

relative growth rates. In this way plants can be grown at sub maximal growth rates but with stable internal nutrient concentrations and no deficiency symptoms.

Seeds were germinated as for the previous experiments but were then inserted into foam disks and placed into the lid of one of three spray culture units. The culture units were as described in Ingestad and Lund (1986) but without automatic pH and conductivity control (Figure 4.1). The nutrient solution contained the same stocks as were used to make the growth solution described above. To allow the plants to become established, the initial solution contained sufficient nutrients to maintain maximum growth (stocks solutions were added to the 5 litre reservoir to give $NH_4^+$ and $NO_3^-$ concentrations of 282 μM and 433 μM, respectively). Once the plants were established (2 weeks), growth rate manipulations commenced. Before nutrient addition treatments commenced, all the plants were weighed and the foam support disks replaced (to remove any N contained in the foam).

***Figure 4.1.*** *Spray culture units used for growing eucalypt seedlings at different relative addition rates. Plants shown were grown at (left to right) 2.5%, 7.5%, and 12% RAR.*

Nutrients were supplied hourly from two stocks kept in separate reservoirs. There was 5 litres of solution circulating in each growth unit. The amount of nutrient

added hourly was set at an exponentially increasing rate (RAR) in line with the expected N concentration of the plants. The RAR treatments were 2.5, 7.5, and 12 %. The 12% RAR treatment was commenced immediately after weighing plants. The units with lower addition rates were put into a lag phase, which is a period during which no nutrients are supplied to the plants and the nitrogen concentration of the plants is allowed to drop (through dilution during growth of new plant tissue) to that corresponding with their treatment. Once the plants were thought to have reached the desired nitrogen concentrations (1-2 weeks), nutrient additions commenced. The lower addition rates were set as 7.5% and 2.5%.

Daily measurements were made throughout the growth period of pH, conductivity and temperature of the circulating solution in each unit. During this period, samples of the growth solution were taken and frozen for $NH_4^+$ and $NO_3^-$ determinations.

Relative growth rates were calculated for plants on which uptake was measured according to Equation 4.1, after plant material had been dried at 80°C for 48 hours and re-weighed.

$$RGR = \frac{\ln w_1 - \ln w_2}{t_2 - t_1} \qquad \text{Equation. 4.1}$$

*where $w_1$ and $w_2$ are plant dry weights (gDW) at times $t_1$ and $t_2$ (days).*

### 4.2.2 Uptake measurements

On the day prior to uptake measurements, plants were removed from the culture units in the glasshouse and brought into the laboratory. Plants were carefully suspended in air and allowed to drip for one minute before being weighed. After weighing, plants were placed in acrylic cylinders (cuvettes), the volume of which enabled the plant volume to solution volume ratio to be approximately 60 mL $gFWroot^{-1}$. A modified hypodermic needle attached to fine silicon tubing was placed in the bottom of each cuvette and air supplied using an aquarium pump. The cuvettes were mounted in a sheet of grey perspex positioned over a temperature controlled water bath (Figure 4.2). The perspex sheet excluded almost all light from the roots whilst foil covers were used to exclude any light coming in around the plant stems. Light was supplied from a 400 W metal halide lamp positioned directly above the

plants such that at the leaf surface the light intensity was approximately 600 μmol $m^{-2}$ $s^{-1}$ (PPFD). The photoperiod was kept the same as that throughout the culture period.

***Figure 4.2.*** *Plants in cuvettes during a depletion experiment. Bases of the acrylic cylinders were in a temperature controlled water bath.*

Once the plants were in cuvettes, the solution was refreshed and plants left overnight. The pre-treatment solution was full nutrient solution diluted so that the initial concentration would, after some depletion overnight, have similar N concentrations as the uptake solutions. At the beginning of uptake measurements the pre-treatment solutions were drained and replaced with uptake solutions. This solution was immediately drained and replaced with fresh solution to mark the start of the measurement. The uptake solution consisted of an N source ($NH_4Cl$, $KNO_3$ or $NH_4NO_3$ depending on the objective), 0.5 mM $CaSO_4$, 5 μM $K_2HPO_4$, and a pH buffer. Apart from the pH experiments, this buffer was 5 mM tartaric acid adjusted to pH 4.2 with NaOH. The phosphate was present to avoid the possibility of short term phosphate deficiency (Smart and Bloom, 1988).

The first sample was taken five minutes after the start of the measurement period, and sampling was continued at regular intervals (≈ 20 min). Samples were 1.3

mL and replaced with an equivalent volume of DI $H_2O$. Samples were frozen immediately after sampling. Water loss through transpiration was replaced at regular intervals.

At the end of the measurement period plants were removed from the cuvettes and once again carefully suspended in air and allowed to drip for one minute before being weighed. The difference between this measurement and the measurement at the start of the experiment was used to estimate plant growth during the measurement period. Plants were then separated into roots and shoots and weighed. Roots were blotted for 1 minute before weighing. After weighing roots were stored in 50% ethanol prior to root length determination. Root lengths were calculated using the modified line intersect method (Tennant, 1975). Root diameters were based on the average of 20 root segments from each root length sample. Shoots were dried at 80°C for 48 hrs prior to weighing.

Depletion curves (C(t)) were constructed from the nitrogen concentration of the samples taking into account dilution due to sampling. Uptake rates were calculated in the same manner as Lee and Rudge (1986), i.e. the maximum slope of the depletion curve during the first four hours of measurement, which, unless otherwise stated, was based on a linear regression of the initial part of the depletion curve. Uptake rates were calculated with respect to both root fresh weight or root surface area.

Kinetic parameters were not estimated for $NO_3^-$ in any of the experiments as uptake rates were so low that solution concentrations were not fully depleted. Kinetic parameters for $NH_4^+$ were estimated only for the RGR/nutrient status experiments and methods used are contained within that section (4.2.2.4). For other experiments $NH_4^+$ C(t) curves were not suitable for an accurate estimation of kinetic parameters (there were not enough points in the region of $K_m$).

#### *4.2.2.1 N source*

To investigate $NH_4^+$ effects on $NO_3^-$ uptake (and vice versa) N was provided in one of three forms; $NH_4NO_3$, $NH_4Cl$, and $KNO_3$. The N concentration was 1 mM, and the solution contained 0.5 mM $CaSO_4$, and 5 µM $K_2HPO_4$. Experiments were carried out on different occasions with solutions buffered to either pH 4.2 or pH 6.1. A pH of 4.2 was maintained using 5 mM tartaric acid adjusted to pH 4.2 with NaOH.

A pH of 6.1 was maintained using 10 mM MES adjusted to pH 6.1 with NaOH. A higher MES concentration (10 mM) than tartaric acid (5 mM) was required because MES is a monoprotic buffer whilst tartaric acid is a diprotic buffer. Each pH, and N source was replicated three times with individual plants. Plants were set up in cuvettes in the laboratory the night before the assay. The overnight pre-treatment solution was half strength nutrient solution (800 μM $NH_4^+$, 1250 μM $NO_3^-$).

Differences between uptake rates for different N sources were separately analysed at each pH using a completely randomised ANOVA design, with differences between means determined by least significant differences ($P = 0.05$). For this purpose there were 3 replicates of 4 treatments: $NO_3^-$-single source, $NH_4^+$-single source, $NO_3^-$-combined source, and $NH_4^+$-combined source.

#### *4.2.2.2 pH effects on N uptake*

For the experiments investigating the effect of pH on uptake rates, the N source was 250 μM $NH_4NO_3$. Solutions also contained 0.5 mM $CaSO_4$, 5 μM $K_2HPO_4$, and either 5 mM tartaric acid for pH 4 or 10 mM MES for pH 6. The pH was adjusted using NaOH. There were 4 plants (replicates) measured at each pH. The plants were set up in cuvettes in the laboratory the night before the assay. The overnight pre-treatment solution was 80 % nutrient solution (1.28 mM $NH_4^+$, 2.00 mM $NO_3^-$).

Differences between uptake rates at different pH and for different ions were analysed using a completely randomised design ANOVA, with differences between means determined by least significant differences ($P = 0.05$). Variances were not homogeneous so uptake rates were log transformed before the analysis.

#### *4.2.2.3 Temperature*

The day prior to uptake measurements for temperature effects, the plants were moved to the laboratory and placed in cuvettes. The cuvettes were placed in different water baths, set at either 10 °C or 20 °C. The overnight pre-treatment solution was quarter strength nutrient solution (400 μM $NH_4^+$, 625 μM $NO_3^-$). At the start of the measurements the 20°C pre-treated plants were assayed at 20°C and 10°C plants at 10°C. The N source was 100 μM $NH_4NO_3$. Solutions also contained 0.5 mM $CaSO_4$, 5 μM $K_2HPO_4$, and 5 mM tartaric acid adjusted to pH 4 using NaOH. At the end of

this first assay the plants were once again supplied with pre-treatment solution and kept at the same temperatures overnight. The following day the plants were assayed again with 100μM $NH_4NO_3$, but just prior to the start of the assay the temperatures of the water baths were switched such that the 20°C cultured plants were assayed at 10°C and the 10°C plants were assayed at 20°C. After this assay the plants were again supplied with pre-treatment solution and kept overnight at the new temperatures. On the third day the plants were again assayed at the new temperatures.

As in other experiments a 1 minute drip weight of the whole plant was taken before and after assays, but in this case this was done at the beginning of the first assay and then again after the third assay. Tissue N concentration was determined on representative plants from each of the 10°C and 20°C cultures.

Differences between uptake rates of plants grown at different culture temperatures and measured at different assay temperatures were analysed using a completely randomised design ANOVA, with differences between means determined by least significant differences ($P = 0.05$).

#### *4.2.2.4 RGR / Nutrient status*

Because plants were grown at different growth rates and reached measurable sizes (≈ 2 g total FW) at different times, it was not possible to measure uptake rates on the different growth rate plants at the same age. The growth rates of the lowest relative addition rate plants was so slow that when uptake rates were measured they were considerably smaller than the plants grown at the higher growth rates.

The overnight pre-treatment solutions for these experiments were dependent upon the RAR treatment and based on the conductivity of the nutrient solutions within the growth units prior to removal of the plants. Conductivity was chosen as a simple measure of the dilution of stock. The pre-treatment solution in a 10 litre reservoir was recirculated through the cuvettes at a rate of 1 mL $min^{-1}$ using a peristaltic pump.

Prior to the start of depletion measurements, the cuvettes were rinsed with 0.5 mM $CaSO_4$ and then the $CaSO_4$ refreshed and left for 20 minutes. At the end of the $CaSO_4$ rinse the cuvettes were filled, drained and refilled with assay solution. The assay solution for all three RAR treatments was 100μM $NH_4NO_3$, 500 μM $CaSO_4$

and 5 μM $KHPO_4$. The assays were run long enough to fully deplete the solutions of $NH_4^+$. The $NH_4^+$ depletion data was then treated as described in Chapter 3. The C(t) data were fitted using 5° polynomials using the polynomial regression function of the computer package Sigma plot (Jandel Scientific). The equations of the fitted curves were then differentiated to calculate the uptake rate at a given concentration. Kinetic parameters were estimated by fitting the revised Michaelis-Menten relationship (Equation 3.1) via the non-linear regression function of the computer package Sigma plot (Jandel Scientific).

Differences between uptake rates expressed on different scales (root weight, length and plant weight), and between tissue concentrations were analysed using a completely randomised design ANOVA, with differences between means determined by least significant differences ($P = 0.05$).

### 4.2.3 Nitrogen determination

#### *4.2.3.1 Solution samples*

Frozen solution samples were thawed immediately before analysis. The $NH_4^+$ and $NO_3^-$ concentrations of samples were analysed using a continuous flow analyser (Autoanalyser II, Technicon). Ammonium was detected via a modified Berthelot indophenol reaction and $NO_3^-$ was detected via hydrazine-copper reduction (Rayment and Higginson, 1992).

#### *4.2.3.2 Plant tissue*

It was not possible to calculate the nitrogen concentration of assayed roots as they were destroyed in the root length measurements. To ascertain the tissue N concentration of the plants used in the experiments, three plants were selected from each growth unit. These plants had their roots rinsed for 1 minute in DI $H_2O$ and were then separated into roots and shoots before weighing. The tissue was then dried at 80°C for 48 hrs. After re-weighing the root and shoot tissue was ground separately to a fine powder using a centrifugal mill. A sub-sample of the ground tissue was then digested using the single acid-hydrogen peroxide method (Lowther, 1980), and the total N concentration determined using continuous flow analysis (McLeod, 1992).

### 4.2.4 Statistical analysis

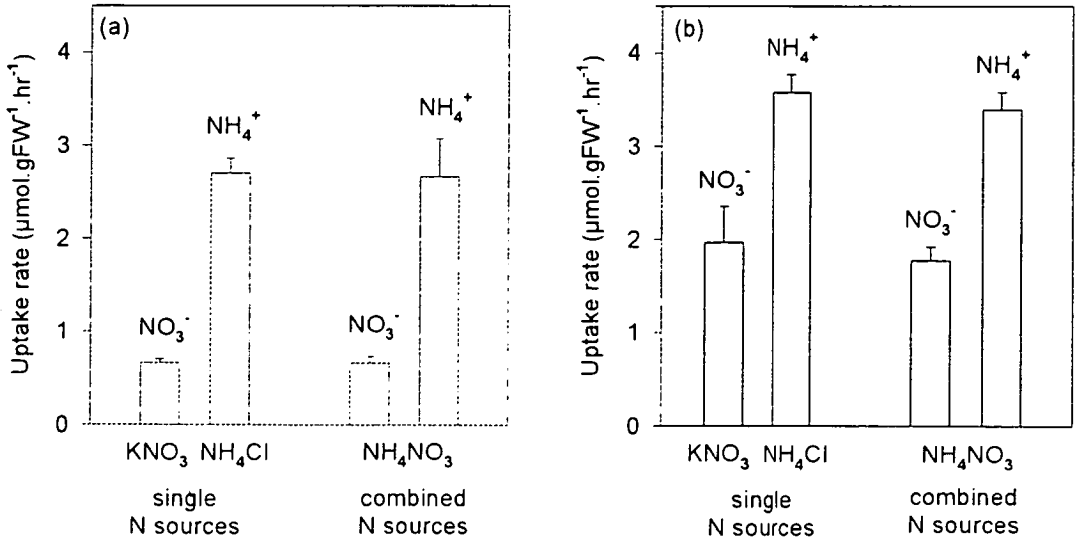
All statistical analyses were carried out using the statistical program SAS (SAS Institute). The SAS procedure ANOVA was used for analysis of variance.

## *4.3 Results*

### 4.3.1 N source

At both pH 6 and pH 4 (Figure 4.1 a. and b.), $NH_4^+$ uptake rates were higher than $NO_3^-$ uptake rates ($P < 0.05$). At pH 6, $NH_4^+$ uptake rates were 4 times higher than $NO_3^-$ uptake rates, whilst at pH 4, $NH_4^+$ uptake rates were almost twice that of $NO_3^-$ uptake rates. Ammonium and $NO_3^-$ uptake rates were the same when N was applied as either a combined or single source at both pH 4 and pH 6 ($P < 0.05$). The uptake rates of both $NH_4^+$ and $NO_3^-$ were less at pH 6 than at pH 4 but as they were measured on separate occasions this difference may have been due to uncontrolled factors such as the age of their root systems.

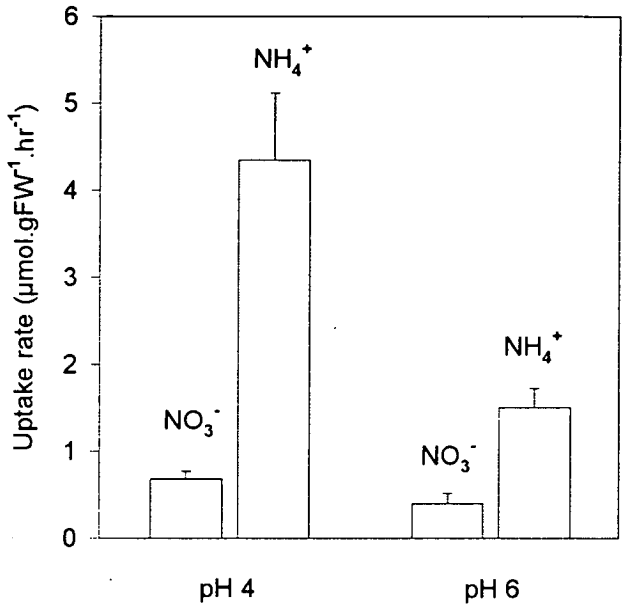
***Figure 4.3.*** *Uptake rates of $NH_4^+$ and $NO_3^-$ with different N sources. Nitrogen was present as either $NH_4NO_3$, $NH_4Cl$, or $KNO_3$. The initial concentration was 1 mM N. (a) pH was buffered to pH 6 with 10 mM MES, and (b) pH was buffered to pH 4 with 5 mM tartaric acid. (Values are means ± SEM, n=3).*



### 4.3.2 pH

Uptake rates of $NH_4^+$ were approximately 3 times higher at pH 4 than pH 6 (Figure 4.4). Average $NO_3^-$ uptake rates were also higher at pH 4 but only by 30% and this difference was not significant at the $P = 0.05$ level.

***Figure 4.4.*** *Uptake rates of $NH_4^+$ and $NO_3^-$ at pH 4 and pH 6 from solution containing 250 µM $NH_4NO_3$ plus 0.5 mM CaSO4. The pH was buffered to pH 4 with 5 mM tartaric acid and to pH 6 with 10 mM MES. (Values are means ± SEM, n=4).*




The effects of pH were similar to those found in the N source experiment (Section 4.3.1) at different pH values (Table 4.2). Although not directly comparable, because they were carried out on different occasions and at different concentrations, the trend shown in the N source experiments of higher uptake rates at lower pH was evident in dedicated pH experiments. Uptake rates were generally lower at 250 µM than at 1 mM, except for $NH_4^+$ uptake at pH 4, which was higher at 250 µM than at 1 mM.

***Table 4.2.*** *Comparison of $NH_4^+$ and $NO_3^-$ uptake rates from solutions at different pH, and N concentrations, and with different N sources. Values are uptake rates (μmoles.gFW$^{-1}$. hr$^{-1}$) ± SEM (n=4). The uptake rates from different N sources at 1 mM were pooled (n=6). Shading (none, light, and dark) indicates experiments carried out on different days. Ammonium uptake as a percentage of total N uptake is also included.*

| | | 1 mM | $NH_4^+$:total N | 250μM | $NH_4^+$:total N |
|---|---|---|---|---|---|
| pH 4 | $NH_4^+$ | **3.48** ± *0.13* | | **4.35** ± *0.77* | |
| | $NO_3^-$ | **1.87** ± *0.19* | 65 % | **0.69** ± *0.08* | 86 % |
| pH 6 | $NH_4^+$ | **2.68** ± *0.20* | | **1.50** ± *0.22* | |
| | $NO_3^-$ | **0.66** ± *0.04* | 80 % | **0.40** ± *0.12* | 78 % |

### 4.3.3 Temperature

#### *4.3.3.1 Plant growth*

After the two-week temperature pre-treatment, plants with roots at 10°C were half the size of the 20°C pre-treated plants and had higher root:shoot ratios (Table 4.3). During the three days of assays there was a change to this ratio as the plants grew. Based on the difference between the 1 minute drip weights at the beginning and end of the assays the 20°C pre-treated plants grew 15 % (± 0.8 *SEM*) during the assays whilst the 10°C pre-treated plants grew 32 % (± 1.9 *SEM*). These differences in growth rates during the experiments led to the 10°C pre-treated plants only weighing 30% less than the 20°C by the end of the assays (Table 4.3).

***Table 4.3.*** *Freshweights and root:shoot ratios of plants with 2-week pre-treatments at 10°C or 20°C. The tissue plants were used for determination of tissue N concentrations (Table 4.4) and were taken from the same cultures as the assay plants. The assay plants had three days of extra growth than the tissue plants during which the root temperatures were changed. Values are means ± SEM.*

| | Plant fresh weight (g) | | Root:shoot ratio | |
|---|---|---|---|---|
| | Tissue plants (*n*=3) | Assay plants (*n*=4) | Tissue plants (*n*=3) | Assay plants (*n*=4) |
| 20°C pre-treated plants | 3.94 ± *0.13* | 6.00 ± *0.51* | 0.92 ± *0.04* | 0.78 ± *0.03* |
| 10°C pre-treated plants | 7.64 ± *0.59* | 8.81 ± *0.88* | 0.83 ± *0.04* | 0.99 ± *0.07* |

Uptake rates were based on the root fresh weight at the end of the three days of assays. Due to growth that occurred during this period, the uptake rates on the first two days were underestimated. The uptake rates of the 10°C pre-treated plants were underestimated more than the 20°C pre-treated plants due to their greater growth during this period. This source of error in estimating uptake rates would not have altered the overall trends shown.

Hot and cold pre-treatments had an effect on the N concentration of the tissue (Table 4.4). The N concentration of the roots in the 20°C pre-treated plants was the same as that in the 10°C plants whilst N concentrations of the shoots were approximately 40% higher.

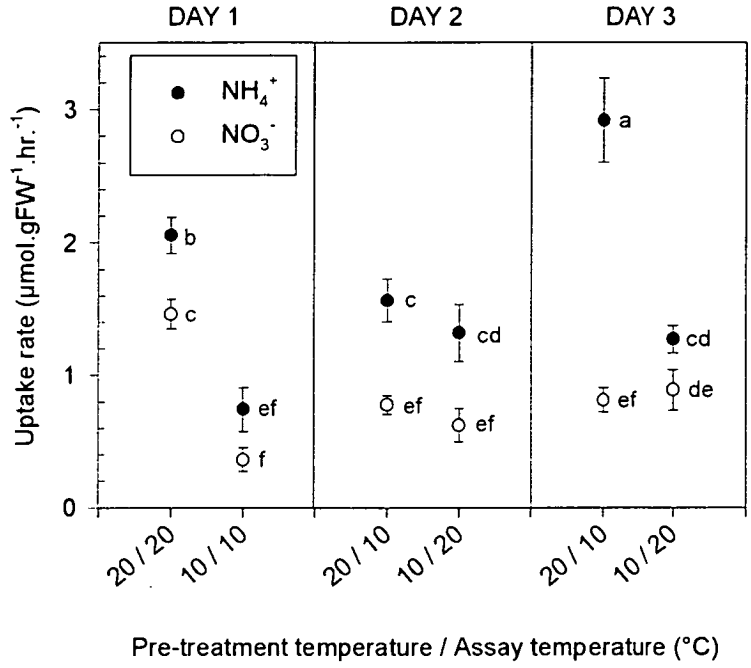
***Table 4.4.*** *Tissue nitrogen concentrations of representative plants taken from cultures pre-treated at 10°C or 20°C. Values are in either mgN.gDW$^{-1}$ or mgN.gFW$^{-1}$. Values are means ± SEM (n=3).*

| | Dry weight basis | | Fresh weight basis | |
|---|---|---|---|---|
| Pre-treatment | Root | Shoot | Root | Shoot |
| 10°C | 36.25 ± *1.57* | 30.83 ± *1.40* | 3.79 ± *0.16* | 9.49 ± *0.43* |
| 20°C | 36.09 ± *0.78* | 42.19 ± *1.42* | 2.66 ± *0.06* | 9.54 ± *0.32* |

### *4.3.3.2 Uptake rates*

Uptake rates for $NH_4^+$ were higher than $NO_3^-$ for all plants, regardless of pre-treatment or assay temperature (Figure 4.5). Plants pre-treated at 20°C and measured at 20°C had higher $NH_4^+$ and $NO_3^-$ uptake rates than plants pre-treated at 10°C and measured at 10°C. When 20°C plants were assayed at 10°C, uptake rates were reduced, but not down the levels of the 10°C pre-treated plants assayed at 10°C. When the plants pre-treated at 10°C were assayed at 20°C the uptake rates increased, but were much less than the 20°C pre-treated plants when assayed at 20°C. On the third day of assays, i.e. the second day at the new temperature, uptake rates were the same as for those on day 2 except for the $NH_4^+$ uptake rate of plants pre-treated at 20°C. For these plants the $NH_4^+$ uptake rate was increased dramatically above that measured on the 1st and 2nd days, and it was 24 hrs since first being changed to solution of 10°C. The uptake rate was almost twice that of the first measurements at 10°C and almost a third higher than that measured at 20°C.

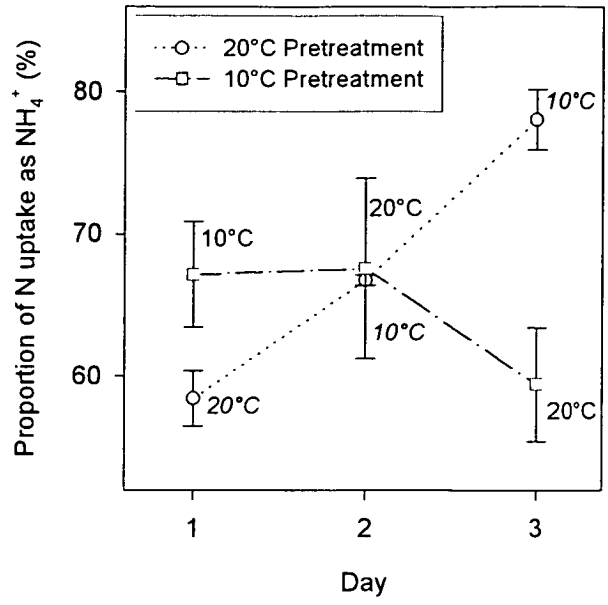
***Figure 4.5.*** *Uptake rates for $NH_4^+$ and $NO_3^-$ measured over three days by plants with roots acclimatised initially to either 20°C or 10°C. On day 1 plants were measured in solutions at the same temperature as the pre-treatment. On day 2, just before the assay, the solution temperature was changed to either 10°C higher or lower than the pre-treatment temperature. After the day 2 uptake measurement, solutions were kept at the new temperatures and the plants were measured again at this temperature on day 3. Values are means ± SEM (n=4). Values with the same letter are not significantly different (P = 0.05).*




The temperature coefficient ($Q_{10}$) was calculated as the ratio of uptake rates measured at pre-treatment temperatures on the first day of assays and then the changed temperatures on the second day of assays. The $Q_{10}$ values for $NH_4^+$ and $NO_3^-$ uptake between 10°C and 20°C were 1.31 and 1.88, respectively for plants pre-treated in 20°C solutions, and 1.78 and 1.72 respectively for plants pre-treated in 10°C solutions.

The proportion of N uptake as $NH_4^+$ or $NO_3^-$ changed between pre-treatment temperatures and between the days of assays when the temperature was changed (Figure 4.6). The trend shown was that $NH_4^+$ uptake as a proportion of N uptake was higher at 10°C than at 20°C.

*Figure 4.6. Uptake of $NH_4^+$ as a proportion of total N uptake over the 3 days of assays. Assay temperatures are shown with each symbol. Values are means (± SEM).*




Uptake rates were calculated as the maximum uptake rates within the first 4 hours of uptake. In most cases the initial depletion could be fitted with a straight line with $r^2$ values generally greater than 0.95. The $NH_4^+$ depletion curves for plants pre-treated at 20°C and then moved to 10°C had a bell shaped initial curve and were not adequately fitted by straight lines. These curves were fitted with quadratic curves and the $r^2$ values were all above 0.95. An example of the bell shape curve is presented in Figure 4.7.

### 4.3.4 RGR and Nutrient status

#### *4.3.4.1 Growth*

Although the RGR of the three treatments did increase with increasing RAR, the RGR of the three treatments did not match the RAR (Table 4.5). As the plants became larger the RAR of the 12% RAR treatment needed to be reduced due to increasing conductivity of the reservoir solution. The increase in conductivity was thought to reflect nutrient additions in excess of plant requirements. Along with the increased conductivity was an increase in $NO_3^-$ concentration, which temporarily reached 1.4 mM, and a decrease in $NH_4^+$ concentration to that which was 20 times less than the $NO_3^-$ concentration (0.07 mM $NH_4^+$). Hence, during this phase, ratios of $NO_3^-$ :$NH_4^+$ increased from 60:40 to 60:3. Also in the 7.5% RAR treatment, $NO_3^-$

***Figure 4.7.*** *Examples of $NH_4^+$ depletion curves on day two of measurements in the temperature experiment. The two sets of points are from different treatments, one pre-treated at 10°C and moved to 20°C (quadratic fit) and the other pre-treated at 20°C and moved to 10°C (linear fit).*

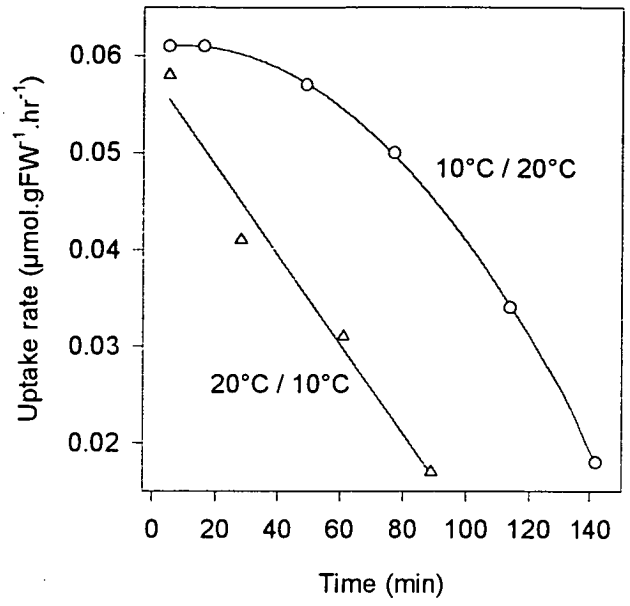
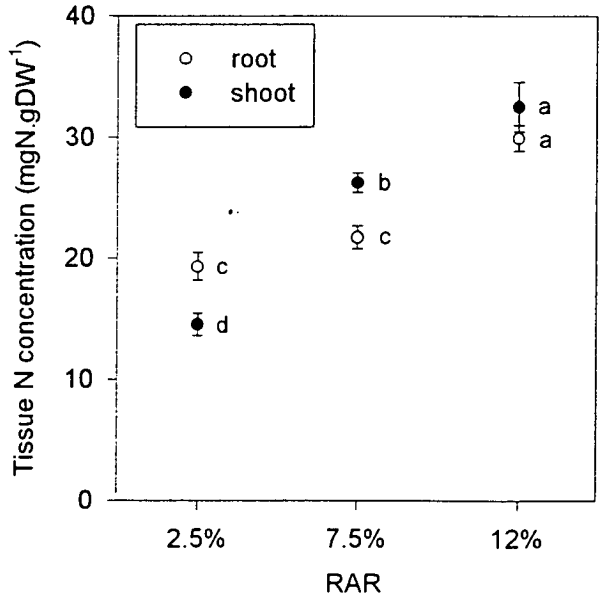



***Figure 4.8.*** *N concentration of plants grown at different relative addition rates. Values are means ± SEM (n=4). Values with the same letter are not significantly different (P = 0.05).*




concentrations (40 µM) remained much higher than $NH_4^+$ concentrations (undetectable), whilst neither $NH_4^+$, nor $NO_3^-$ were detectable in the 2.5% RAR treatment.

Although the differences in RGR did not reflect the magnitude of the RAR differences, other plant characteristics were markedly different between RAR treatments. For example, the N concentration of both roots and shoots increased with increasing RAR (Figure 4.8). In the two highest RAR treatments, root N concentrations were lower than the shoot concentrations but in the 2.5% RAR the shoot concentration was lower. As well as the effect on tissue N concentration, different RAR treatments affected other growth characteristics (Table 4.5). For example, on a fresh weight basis, the three different RAR plants had a greatly different proportion of roots. The 12% RAR plants had roots less than half the mass of the shoots whilst the 2.5% RAR plants had bigger roots than shoots (Table 4.5, Figure 4.13). On a dry weight basis this relationship was less well defined with the 7.5% and 2.5% treatments being indistinguishable, and a third greater than the 12% RAR treatment.

On a whole plant basis, the fresh weight:dry weight ratios of the 12% and 7.5% RAR treatments were the same, but for the 2.5% RAR plants, the ratio was slightly but significantly lower. The whole plant values did, however, hide some important differences between treatments. The fresh weight:dry weight ratios of the roots were similar for all treatments. Where as the fresh weight:dry weight ratios for the shoots were similar for the 12% and 7.5% RAR treatments, but much lower for the 2.5% RAR treatment.

***Table 4.5.*** *Growth characteristics of plants grown at different relative addition rates (FW freshweight, DW dry weight). RGRs are mean values ± SEM (n=8). Ratios are means of ratios for individual plants (± SEM, n=4).*

| RAR ($d^{-1}$x100) | *2.5* | *7.5* | *12* |
|---|---|---|---|
| RGR ($d^{-1}$x100) | 5.76 ± *0.28* | 6.80 ± *0.19* | 7.67 ± *0.23* |
| root:shoot (FW) | 1.16 ± *0.06* | 0.73 ± *0.05* | 0.42 ± *0.05* |
| root:shoot (DW) | 0.33 ± *0.02* | 0.33 ± *0.02* | 0.22 ± *0.02* |
| FW:DW (whole) | 3.74 ± *0.06* | 4.57 ± *0.13* | 4.73 ± *0.73* |
| FW:DW (root) | 8.25 ± *0.55* | 7.86 ± *0.41* | 7.73 ± *1.25* |
| FW:DW (shoot) | 2.31 ± *0.12* | 3.51 ± *0.11* | 4.06 ± *0.61* |

### *4.3.4.2 Uptake rates*

When uptake rates were calculated via linear regression of the initial part of the depletion curves, $NH_4^+$ uptake rates were higher than $NO_3^-$ uptake rates for all three RAR treatments (Figure 4.9 a, b). The proportions of $NH_4^+$ uptake to $NO_3^-$ uptake did, however, differ. Ammonium as a proportion of total N uptake was 74%, 85%, and 86% for the 2.5%, 7.5% and 12% RAR plants, respectively.

For $NH_4^+$ uptake on a fresh weight basis, plants with 7.5% RAR had the lowest uptake rates and there was no difference between the 12% and 2.5% RAR plants (Figure 4.9 a). The 2.5% RAR plants had $NO_3^-$ uptake rates that were twice those found for the 7.5% and 12% RAR plants. On a surface area basis, uptake rates of the different RAR plants were similar to those on a weight basis (Figure 4.9 b), except for the 2.5% RAR plants where the uptake rates were less than the higher RAR plants. This difference was due to the increased root surface:weight ratios of the 2.5% RAR plants (root diameters ± *SEM (n=80)*: 2.5% RAR, 10.94 ± *0.84*; 7.5% RAR, 12.36 ± *0.84*; 12% RAR, 13.94 ± *1.10*).

***Figure 4.9.*** *Ammonium and $NO_3^-$ uptake rates for plants grown in different RAR treatments. Uptake rates shown are the maximum uptake rates within 4 hours of the start of the experiment: (a) in terms of the root fresh weight, and (b) in terms of the root surface area. Values are means ± SEM (n=4). Values with the same letter are not significantly different (P = 0.05).*

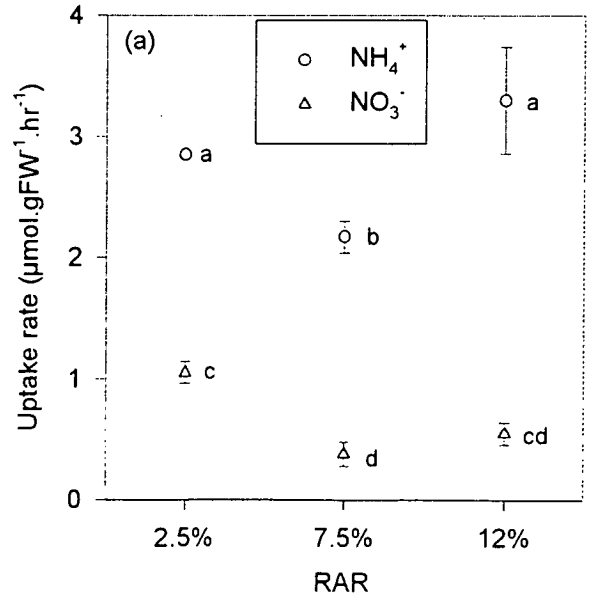
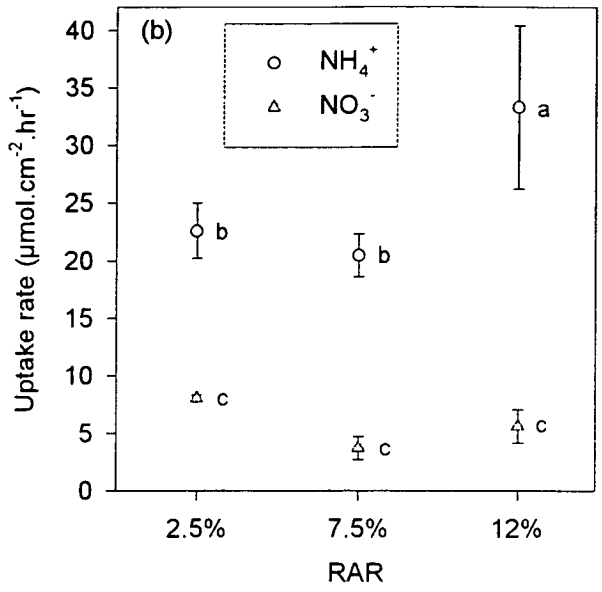
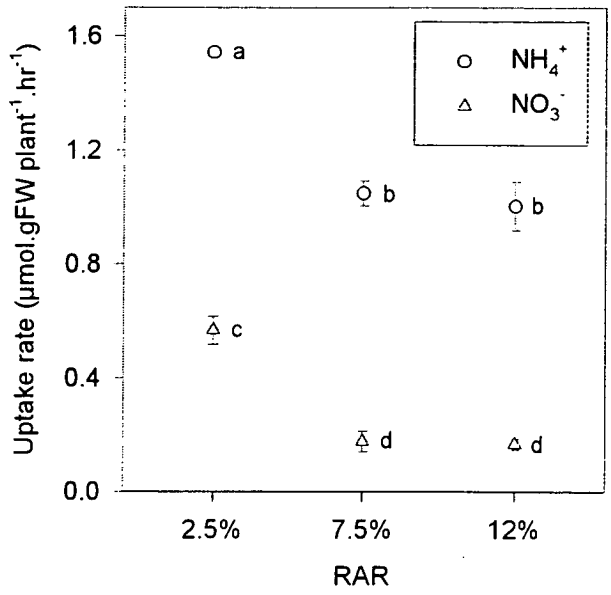


***Figure 4.9. (cont.)*** *Ammonium and $NO_3^-$ uptake rates for plants grown at different RAR. Uptake rates shown are the maximum uptake rates within 4 hours of the start of the experiment: (a) in terms of the root fresh weight, and (b) in terms of the root surface area. Values are means ± SEM (n=4). Values with the same letter are not significantly different (P = 0.05).*




Although the rates of uptake are similar across RAR treatments on a unit of root basis, this does not relate to the size of the whole plant due to the large differences in root:shoot ratios. When uptake rates are calculated on a plant weight basis the differences between treatments were greater, and uptake rates were inversely related to RAR (Figure 4.10).

***Figure 4.10.*** *Ammonium and $NO_3^-$ uptake rates for plants grown at three different RAR. Uptake rates shown are the maximum uptake rates within 4 hours of the start of the experiment in terms of the whole plant fresh weight. Values are means ± SEM (n=4). Values with the same letter are not significantly different (P = 0.05).*




Nitrate uptake rates were linear over the range of depletion and were sufficiently low that the concentration was only depleted to 50 µM, from an initial concentration of 100 µM. Hence, values of $K_m$, $V_{max}$ and $C_{min}$ could not be estimated for $NO_3^-$ uptake. Ammonium depletion curves were fitted using 5° polynomials (Refer Chapter 3). For the 2.5% RGR plants, the $NH_4^+$ concentration had not levelled off by the end of the depletion period (10 hours) by which time it had decreased to 10 µM. Hence, no estimates of $K_m$ could be made for these plants but it is assumed that they would have to be at, or below, this concentration of 10 µM.

Without modification, the V(C) data produced for the 7.5% and 12% RAR plants (Figure 4.11) did not follow the M-M relationship (Figure 3.1). Before estimating kinetic parameters the V(C) data were modified by removing points in the way described in Chapter 3 (Figure 4.12). Estimated kinetic parameters are presented in Table 4.6. Although the resulting curves could be fitted to the M-M equation, the kinetic parameters estimated from these curves seem rather meaningless (Table 4.6). For the 12% RAR plants, the curves had not reached an asymptote within the concentration range and this resulted in high values and errors for the $K_m$ and $V_{max}$ estimates. With modification, the 7.5% RAR curves could be fitted to the M-M relationship, but only by using estimates of $C_{min}$ which were much higher than the measured $C_{min}$ values (estimated 20 µM, measured 1.5 µM).

***Figure 4.11.*** *V(C) data for $NH_4^+$ calculated from depletion experiments with plants grown at RAR of (a) 7.5% and (b) 12%. Curves were produced by first fitting the depletion curves with 5th order polynomials and then using the differentiated polynomials to calculate uptake rates at each concentration point. Different symbols represent the 4 replicates for each of the two RGR treatments.*

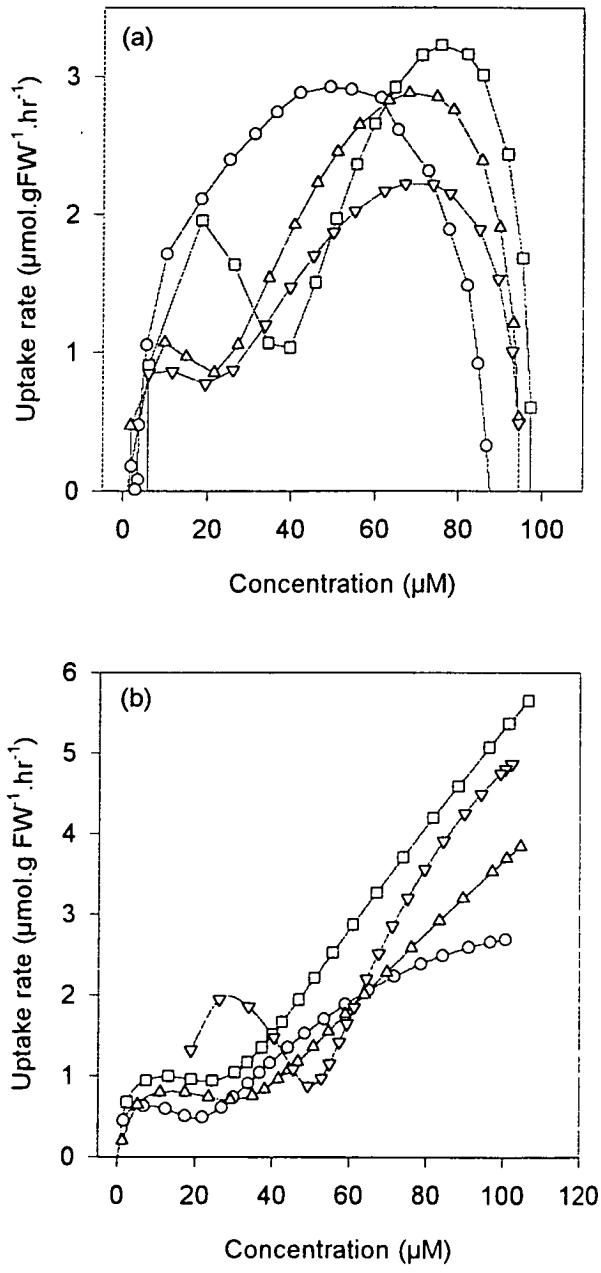


***Figure 4.12.*** *Modified V(C) data that was used to estimate kinetic parameters for (a) 7.5% RAR plants, and (b), 12% RAR plants. The points are estimated uptake rates, whilst the lines are the fitted M-M functions. Different symbols represent the different replicates for each of the RGR treatments.*

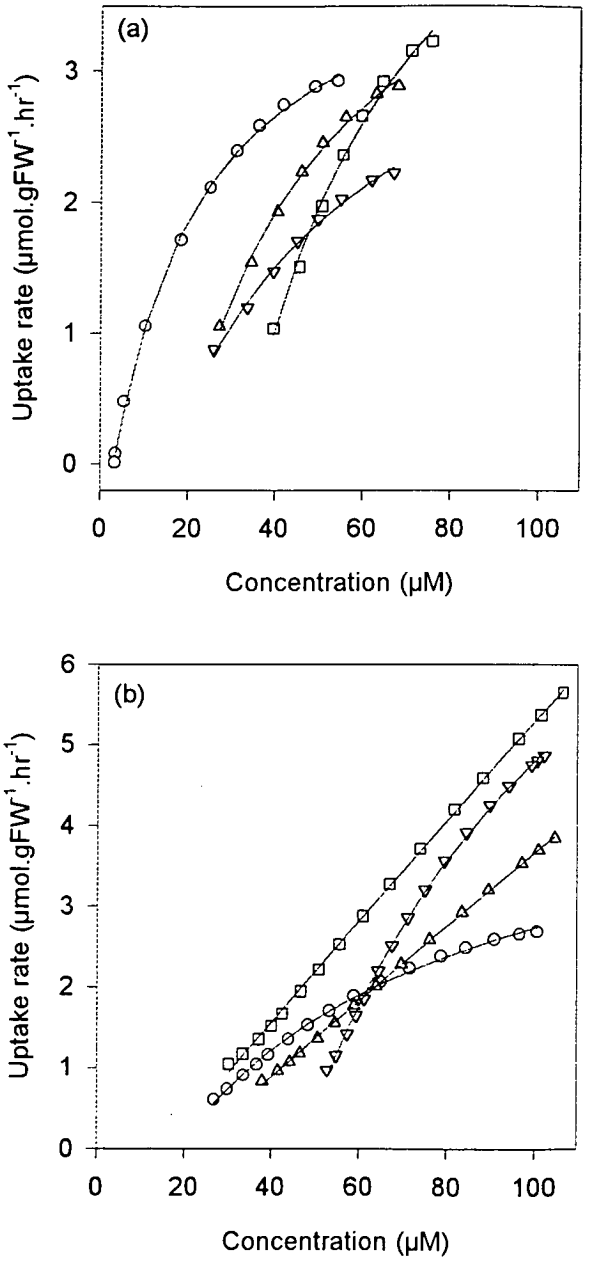
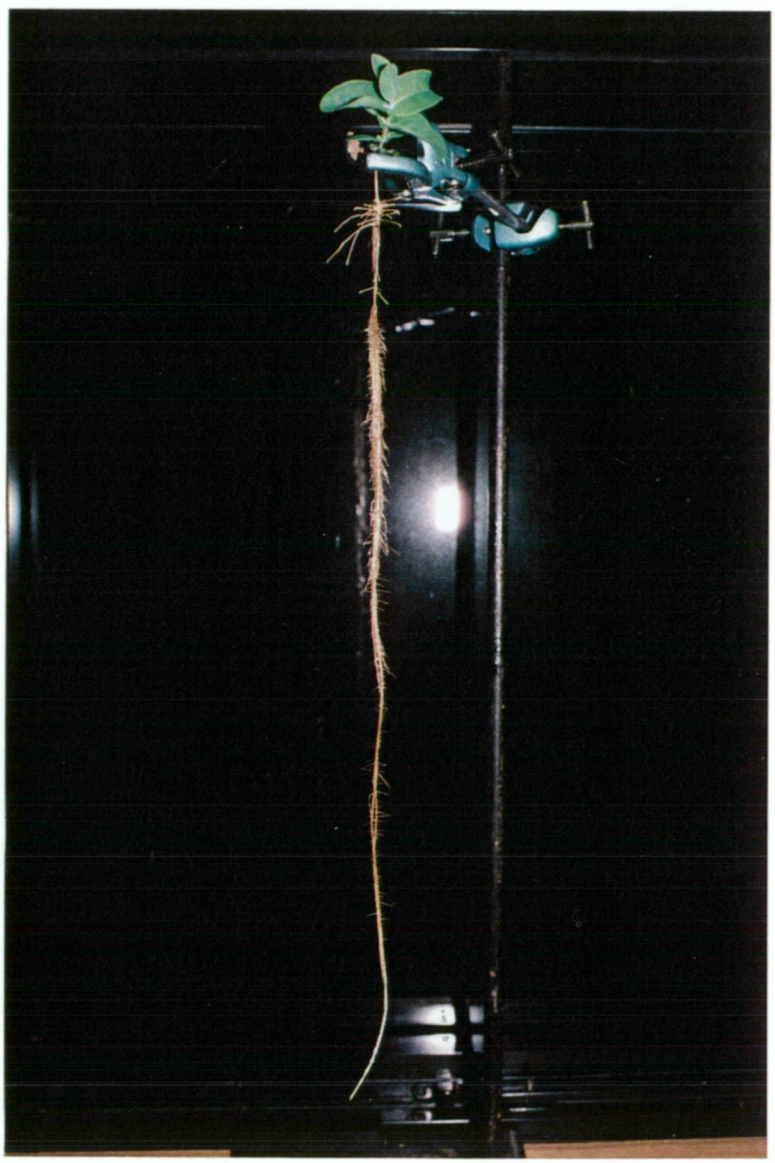


***Table 4.6.*** *Uptake kinetic parameters for $NH_4^+$ estimated from the modified V(C) data of Figure 4.12. Estimates are the means of 4 plants (± SEM), but are unreliable for reasons presented in the discussion.*

| | 7.5% RAR | 12% RAR |
|---|---|---|
| $K_m$ (µM) | 35.5 ± *5.7* | 2330.1 ± *1612.5* |
| $V_{max}$ (µmol $gFW^{-1}$ $hr^{-1}$) | 4.9 ± *0.5* | 141.2 ± *102.0* |
| $C_{min}$ (µM) | 17.1 ± *6.1* | 25.5 ± *7.2* |

***Figure 4.13.*** *Typical plant from the 2.5% RAR treatment. The length of the root system in the picture was approximately 75 cm.*



## *4.4 Discussion*

### 4.4.1 N source

Whether at pH 4 or pH 6 the presence of $NH_4^+$ had no effect on $NO_3^-$ uptake (Figure 4.3 (a) and (b)). These data suggest that $NH_4^+$ does not inhibit $NO_3^-$ uptake by *E. nitens* under these conditions. According to Lee and Drew (1989), the inhibition of $NO_3^-$ uptake by $NH_4^+$ is reduced or even removed when plants are grown on mixed N sources, as were the *E. nitens* plants in this study. Hence, the possibility remains that $NH_4^+$ inhibition of $NO_3^-$ uptake might occur in *E. nitens* if it were grown on only $NO_3^-$ as the N source.

### 4.4.2 pH

Uptake of $NH_4^+$ was much higher at pH 4 than pH 6 (Figure 4.4). Whereas, $NO_3^-$ uptake was not significantly affected by pH. For this reason, $NH_4^+$ as a proportion of uptake was dramatically increased by decreasing pH from pH 6 to pH 4.

Plants were grown at pH 4 and measured at pH 4 and pH 6, so higher uptake rates of $NH_4^+$ at pH 4 than at pH 6 may have been due to adaptation to the culture conditions. Marcus-Wyner (1983) measured uptake of $NH_4^+$ uptake by tomato at pH 4.5, 5.5, and 6.5 after being grown at pH 5.5. Uptake of $NH_4^+$ was found to increase with increasing pH whilst uptake of $NO_3^-$ increased with decreasing pH. Wang et al. (1993b) grew rice plants at pH 6 and then measured $NH_4^+$ uptake at pH ranging from pH 3 to 9. Although uptake was least at pH 3, there was no difference in uptake rates between pH 4.5 and pH 6. Spruce grown at pH 4.5 showed only half the $NO_3^-$ uptake rate when measured at pH 4.5 compared to that when measured at pH 5.5 (Peuke and Tischner, 1991). Hence, results with other species do not support the hypothesis that the higher uptake rates of $NH_4^+$ and $NO_3^-$ at pH 4 for *E. nitens* simply reflected adaptation to the culture pH.

Another possible problem with these results is that pH was maintained using different buffers. It is not possible to use the same buffer to maintain pH at pH 4 and pH 6. For example, MES has a $pK_a$ of 6.15, to have the same buffer capacity at pH 4 as at pH 6.15 would require 100 times the concentration of MES used at pH 6.15. At pH 6.15, 10 mM MES was required to maintain pH in these experiments so pH 4

would have required 1000 mM MES, which would have been an unrealistically high ionic strength. For this reason, tartaric acid ($pK_a = 4.13$) instead of MES was used at pH 4. There have been several cases reported where MES has inhibited plant processes (Medeiros et al., 1993; Nicholas and Harper, 1993). Hence, the possibility remains that physiological responses to the two different buffers may have been the cause of the differences in uptake rates at pH 4 and pH 6.

Carrodus (1969) found that for *E. obliqua*, $NH_4^+$ uptake was 50% higher at pH 6 than pH 4. This result does not agree with that found here. A big difference between the two experiments is that the concentration used in the Carrodus experiments was 10 mM, 10 times the highest concentration used here, and the increased uptake at higher pH may be due to diffusion of $NH_3$ (Smith and Walker, 1978; MacFarlane and Smith, 1982) rather than increased uptake of $NH_4^+$. Ammonium in solution is a combination of the cation ammonium ($NH_4^+$), and the free base ammonia ($NH_3$), in proportions dependent upon the pH (pKa of 9.25). Neutral, small, and polar, $NH_3$ has a higher membrane permeability than $NH_4^+$ (Kleiner, 1981, Ritchie, 1987). With an $NH_4Cl$ concentration of 10 mM at pH 6, the concentration of $NH_3$ in solution is about 5 μM (c.f. 0.05 μM at pH 4). Hence, the increase in $NH_4^+$ uptake with increasing pH found by Carrodus may have reflected the increasing ammonia ($NH_3$) concentration in the external solution.

Results of the pH experiment (Figure 4.4) differ from those inferred from the N source experiments conducted at similar pH (Table 4.2) where the proportion of N taken up as $NH_4^+$ was less at pH 4 than at pH 6. In the two experiments uptake was measured at different initial concentrations (250 μM and 1 mM). At these two concentrations it was assumed that two different uptake systems operate, the HATS and the LATS (Chapter 2, and Introduction to Chapter 3). Hence, I speculate that because the experiments measured uptake at different concentrations the apparent effect of pH reflected two different uptake mechanisms in these concentration ranges.

The $NH_4^+$:$NO_3^-$ ratio (40:60) of the growth solution would normally be expected to maintain a relatively stable pH. Generally $NH_4^+$ is added to nutrient solutions because the combined nitrogen sources lead to a stable pH (Hewitt, 1966). For these cultures the pH of the nutrient solutions, if left unadjusted, dropped to as low as pH 3 in pilot studies with no apparent ill affects on the plants. Hence, constant pH

adjustment was required throughout the culture period and it was also necessary to use 10 mM buffer strength in the uptake experiments.

There are two possible reasons why plants adapted to acid soils might prefer $NH_4^+$ as their N source. Firstly, assimilation of $NH_4^+$ is energetically more efficient than that of $NO_3^-$ (Raven and Smith, 1976). Secondly, nitrification is generally slowest in acid soils and $NH_4^+$ availability in such soils is generally much higher than that of $NO_3^-$ (Runge, 1981). Results in this chapter show that *E. nitens* has a high potential to acidify its environment and a preference for $NH_4^+$ over $NO_3^-$.

### 4.4.3 Temperature

#### *4.4.3.1 Growth and tissue concentration*

The most common effect of low root temperatures on plant growth is reduced total plant growth and higher root:shoot ratios (Clarkson and Warner, 1979; Clarkson et al., 1986; Macduff et al., 1987; Clarkson et al., 1992). These effects were manifest in *E. nitens* when roots were exposed to low temperatures during the two week pre-treatments (Table 4.3). The growth response to changing temperatures was found to be rapid, i.e. plant characteristics changed noticeably during the 3 days of assays, of which only approximately 30 hours was at the new temperature (c.f. tissue and assay plants; Table 4.3).

Nitrogen concentration of the shoots of the 20°C pre-treated plants were lower than that of the shoots (Table 4.4), whilst the root N concentration was the same in both 10°C and 20°C pre-treated roots. This result agrees with that found for other species which showed a reduction in shoot N concentration on a dry weight basis when roots were cooled with respect to shoots (Clarkson et al., 1986; Macduff et al., 1987; Macduff and Jackson, 1991). The low tissue N concentration of the shoots of the low temperature pre-treated plants is thought to reflect lower translocation from the roots to the shoots.

#### *4.4.3.2 Uptake effects*

When measured at 10°C and 20°C, regardless of pre-treatment temperatures, $NH_4^+$ uptake rates were higher than $NO_3^-$ uptake rates. The short term effects of changing the temperature were similar for both $NH_4^+$ and $NO_3^-$ and this is reflected in

the $Q_{10}$ values. The $Q_{10}$ value is simply the ratio of a process measured at two temperatures 10°C apart and is an indication of the temperature dependence of a process. The $Q_{10}$ values between 10°C and 20°C found here (1.31-1.88) are similar to those found for $NH_4^+$ and $NO_3^-$ uptake by rye grass between 15°C and 25°C (1.16-1.47) (Clarkson and Warner, 1979), and for $NH_4^+$ influx in rice between 10°C and 20°C (1.68) (Wang et al., 1993b). The $Q_{10}$ values measured here are not actually those for uptake in terms of nutrients moving from the external solution into the root. As pointed out by Wang et al. (1993b), long term uptake measurements, as measured here, are actually measurements of combined uptake and assimilation. The fact that the $Q_{10}$ values are very similar to those of Wang et al. (where short term influx was measured) suggests that the uptake and assimilatory processes were similarly affected.

When measured at 20°C, the uptake rates of $NH_4^+$ and $NO_3^-$ by 10°C pre-treated plants were considerably less than for plants pre-treated at 20°C. There was not such a relatively large increase in uptake capacity due to low temperature pre-treatments as that found by others (Clarkson and Warner, 1979; Deane-Drummond and Glass, 1983; Macduff et al., 1994). Whereas, there did appear to be evidence of acclimation in the 20°C plants when there was a dramatic increase in the uptake rate of $NH_4^+$ on the third day of measurement for plants pre-treated at 20°C (Figure 4.5). On the third day of assays, the second day at 10°C for these plants, the $NH_4^+$ uptake rate almost doubled that of the previous day and was almost a third higher than when measured at 20°C on the first day.

The first day at 10°C for the 20°C pre-treated plants resulted in reduced uptake rates (Figure 4.5). As evident in the change in root:shoot ratios between pre-treatment and end of the assays (Table 4.3), the lower temperature also reduced root growth compared with shoot growth. Hence, the higher uptake rate on day 3 may represent an adaptation to lower temperature to meet increased demand and an initial reduction in uptake capacity. If uptake had been measured on the 4th day with the 20°C plants again returned to 20°C then it is possible that they would have shown increased uptake rates of $NH_4^+$ compared to pre-treatment values similar to that found in other species (ryegrass, Clarkson and Warner, 1979; barley, Deane-Drummond and Glass, 1983; rape, Macduff et al., 1994, Bigot and Boucard, 1994).

The differences in uptake by the 20°C and 10°C pre-treated plants over the three days of measurement is consistent with a two-phase growth response to changing root temperatures (Clarkson et al., 1986; Macduff and Jackson, 1991). According to this hypothesis, when plant roots are first exposed to low temperature, root growth is slowed but shoot growth continues as before, this corresponds to phase 1. During phase 2, the shoot growth is reduced until a new steady state root:shoot ratio is found and then maintained through further growth. Uptake rates are likely to reflect these different growth phases. During phase 1, uptake will increase to meet increased demand, but during phase 2 demand will be reduced and hence, uptake will also be reduced. According to this proposition, the plants at the start of the assays were in phase 2 before switching to phase 1 when the root temperatures were changed.

The preference for $NH_4^+$ over $NO_3^-$, which was greater at 10°C than at 20°C (Figure 4.6), concurred with similar results for a number of other species at low temperatures (Lyklama, 1963; Clarkson and Warner, 1979; Macduff and Hopper, 1986, Clarkson et al., 1986; Macduff and Wild, 1989). However, the difference in response on day 2 of assays between the plants pre-treated at 10°C and 20°C (Figure 4.6) was suprising. A 10°C decrease in temperature led to an immediate increase in proportion of $NH_4^+$ absorbed in the plants pre-treated at 20°C, whereas, the plants pre-treated at 10°C and changed to 20°C on day 2 showed the same proportion of $NH_4^+$ absorbed as on day 1 when measured at 10°C. Differences in demand could be the reason for this difference in response. The plants pre-treated at 20°C and then changed to 10°C would, it is assumed, have demand great enough such that efflux was insignificant and direct effects on N influx were apparent. Whereas, plants pre-treated at 10°C and then transferred to 20°C would, again it is assumed, have an uptake capacity greater than demand and perhaps N taken up excess to demand was returned to solution as efflux. This efflux could obscure direct effects on N influx since net (not gross) influx was measured. On day 3, the second day at temperatures different to pre-treatment, demand in the plants pre-treated at 10°C would have increased and the differential effects of temperature on $NH_4^+$ and $NO_3^-$ uptake were evident.

The difference in the shape of the depletion curves of the plants given different pre-treatments (Figure 4.6) is hard to explain. Lee and Rudge (1986) found similar

shaped depletion curves when measuring $NO_3^-$ uptake in barley. They suggested that the initial slow rate of uptake could be due to a continuation of efflux when the roots were changed from high concentration (1.5 mM) to 200 μM solutions. If a similar effect occurred in Figure 4.7 (pre-treatment was 600 μM $NO_3^-$, 400 μM $NH_4^+$; assayed in 100 μM $NH_4NO_3$) then both the 20°C and 10°C treatments should have shown this response. Low temperature has been found to stimulate efflux (Bieleski and Ferguson, 1983) but in this case the low temperature plants had been changed to higher temperature. The bell shape may be caused by increased efflux if supply from uptake was suddenly greater (due to the increase in temperature leading to higher activity of uptake mechanisms) and above plant requirements. If this was the case, then it would seem likely that this component would have been reduced on the third day of assays in a similar way that the 20°C pre-treated plants increased rates of uptake of $NH_4^+$on the third day of assays. However, the bell shape was apparent on the third day of assays so this hypothesis is questionable.

### 4.4.4 RGR and Nutrient status

#### *4.4.4.1 Growth*

The RGR did not match the RAR for either of the three RAR treatments (Table 4.5). Similar results have been found in barley, where the RGR exceeded the RAR at lower RAR, but was less than the RAR at higher RAR (Mattsson et al., 1991). In the present case the reason for this lag is thought to be due to inappropriateness of the nutrient solution. The nutrient solution used was developed for spruce (Ingestad and Lund, 1986) and not eucalpyts so there was a rather simplistic assumption made that eucalypts would require similar ratios of nutrients. The basis of the Ingestad culture system is that nutrients used are supplied in the same ratio as they are required. With exponential increases, any differences in the supply ratio from the plants requirements could lead to an accumulation in the circulating solution of unutilised nutrients. In this case, if the optimum required ratio of nutrients was 60:40 $NH_4^+$:$NO_3^-$ (for example), and not 40:60, then this would result in an accumulation of $NO_3^-$ in the reservoir as was found. The same nutrient solution had, however, been successfully used in RAR experiments with the same culture units for growing *Eucalyptus grandis* (Cromer and Jarvis, 1990). It is possible that *E. grandis* had different preferences for $NH_4^+$ and $NO_3^-$

than those found here for *E. nitens* as has been shown between other eucalypt species (Moore and Keraitis, 1971). Also in contrast to the present study, barley plants grown at a growth-limiting RAR (65% of maximum), showed no differences in uptake or growth characteristics between treatments consisting of a range of $NH_4^+$:$NO_3^-$ ratios (Samuelson et al., 1995). Another possibility is that both $NH_4^+$ and $NO_3^-$ were supplied surplus to requirements and high nitrification rates in the recirculating system led to high $NO_3^-$ concentrations (Padgett and Leonard, 1993, Smart et al., 1995).

Plants grown at different RAR showed the characteristics of plants grown using this approach (Larsson et al., 1992; Marschner, 1995), including different root:shoot ratios, tissue N concentration, and shoot fresh weight:dry weight ratio. The largest difference between plants in the RAR treatments was in the root:shoot ratios where the 2.5% RAR plants had very large root systems relative to shoots. This increased relative size of the root system was a classical response to low nutrient availability (Chapin, 1988).

#### *4.4.4.2 Nitrogen uptake*

In terms of root fresh weight, the uptake rates did not change in a consistent relationship with the RAR. The 2.5% RAR plants had $NH_4^+$ uptake rates not significantly different from the 12% RAR plants, whilst their $NO_3^-$ uptake rates were more than twice that of the 12% RAR plants (Fig 4.9 a). The N demand of plants is dependent on RGR (Clement et al., 1978a, 1978b; Clarkson et al., 1986) and would have been lowest in the 2.5% RAR treatment. However, in these other cited experiments, RGR was controlled by either light or temperature and for which there was no difference in RGR due to differences in concentration of nutrients in solution. However, in these experiments only steady-state, long-term uptake was measured. As pointed out by Touraine et al. (1994) for $NO_3^-$ uptake, actual uptake rates are generally lower than the uptake capacity of the plant. Hence, the uptake rates evident in Figures 4.9 and 4.10 could have been more influenced by uptake capacity than demand.

The uptake rates measured in experiments reported in this chapter should be comparable to those of Mattsson et al. (1991), where short term uptake was measured after growing plants in different RAR treatments. Mattsson et al. (1991) found that $V_{max}$ was highest for plants growing at a slightly limiting RAR, and less at

higher and lower RAR. If initial uptake rates were equated to $V_{max}$, then this was not the case in the eucalypt experiments, as shown in Figures 4.9 and 4.10. There were however, differences between the measurement methods. Mattsson et al. (1991) measured uptake as short term decrease in solution concentration in a range of solutions with increasing concentrations, the full range of which was measured in approximately 1 hour. Uptake rates for eucalypts were measured as depletion of a single concentration over 10 hours. As discussed in Chapter 3, different methods of measuring uptake can produce widely different parameter estimates. The differences between the Mattsson et al. (1991) results, and the eucalypt results may be due to this difference in methods.

Plants subjected to a reduction in nutrient availability show increased uptake capacity when nutrients were re-supplied (Lee, 1982; Drew et al., 1984; Lee and Rudge, 1986; Oscarson et al., 1987; Morgan and Jackson, 1988a, 1988b; Siddiqi et al., 1989). It is possible that the increase in uptake rates shown by the 2.5% RAR plants, relative to the 7.5% and 12% RAR treatments, is evidence of this effect.

The preference for $NH_4^+$ as an N source was maintained in all three growth rates. The increase in $NO_3^-$ uptake as a proportion of total N uptake shown by 2.5% RAR plants in Figure 4.9 could have been an adaptation to increased total N uptake in response to low $NH_4^+$ availability. A problem with this assertion is the confounding brought about by the $NO_3^-$ accumulation in the 7.5% and 12% RAR culture units. If plants were constantly exposed to high concentrations of $NO_3^-$, then the reduced uptake could be related to negative feedback activity on the $NO_3^-$ uptake system caused by the high $NO_3^-$ concentrations (Lee and Rudge, 1986; Teyker et al., 1988; Siddiqi et al., 1989; Jackson and Volk, 1992). This hypothesis is supported by the high proportion of $NH_4^+$ taken up in this experiment compared with other experiments in this chapter. Ammonium uptake as a proportion of total N uptake was very high in the 7.5% and 12% RAR treatments (85% and 86%, respectively) whilst in the 2.5% RAR treatment the ratio (74%) was closer to that found in other experiments in this chapter.

The shapes of the unmodified depletion curves (Figure 4.11 a, b) did not follow a rectangular hyperbola characteristic of the M-M relationship. This non M-M shape was also found with the $NO_3^-$ datasets of Laine et al. (1993) and Swiader and Frieji

(1996) presented in Figure 3.3. Based on this limited set of examples, the results here are not atypical for nitrogen. Unlike these other nitrogen datasets, the $NH_4^+$ datasets here were not easily modified to make them fit the M-M relationship.

When V(C) data were modified to fit the M-M relationship the estimated parameters were out of the expected range for $NH_4^+$ found in the literature (Table 3.1), and beyond the concentration ranges measured in this chapter. For the 12% RAR plants the estimates were very high, and suggest that $V_{max}$ values would have been reached at a concentration well above the maximum concentration used here, and led to corresponding $K_m$ values that were greater than 50μM. Parameters estimated for the 7.5% RAR plants were also high but the estimated $K_m$ value was within the concentration range of the experiment (Figure 4.12).

The estimates in the literature suggest that, for most species, the $K_m$ for $NH_4^+$ is generally less than 40μM (Table 3.1). The $K_m$ estimates shown here are much higher than this. It is possible that *E. nitens* roots have much higher $K_m$'s than these other mostly non-tree species, but this seems very unlikely. Kronzucker et al. (1996) found that another tree, spruce, had a $K_m$ for $NH_4^+$ influx of between 20 and 40 μM. The $V_{max}$ values were not easily estimated from the V(C) data. The best estimate of $V_{max}$ is perhaps the flux rates based on linear regression of the initial part of the uptake curve as presented in Figure 4.9 and discussed earlier. Unfortunately, as shown from the fits of the M-M function to the V(C) data (Figure 4.12), $V_{max}$ was not reached in the 1-100 μM concentration range. Hence, these estimates of M-M parameters, especially for the 12% RAR plants, are not reliable.

Even if the absolute values of the $K_m$ estimates for $NH_4^+$ are probably wrong, there appeared to be consistent relative differences between the three RAR treatments, such that the $K_m$ for the three treatments increased with increasing RAR. This interpretation assumed that the $K_m$ for the 2.5% RAR plants was less than 20μM, for the 7.5% RAR plants was approximately 95 μM, and for the 12.5% RAR plants was >100μM.. These differences in $K_m$ would agree with the initial proposition that plants with a lower RGR:nutrient status have a higher affinity for $NH_4^+$.

One reason for the non M-M nature of the concentration dependence shown here could lie in the time span of the depletion experiments. Claassen and Barber (1974) recommended that the solution to root ratio should be such that the

experiments can be completed in 3-5 hrs in order to minimise diurnal changes in fluxes. As the experiments were carried out within the daylength that the plants had been grown in, it seems unlikely that the diurnal effects could have been very large in this case. Apart from the possible diurnal effects on uptake there are other factors that could confound the results of such long-term measurements. Jackson and Volk (1992) subjected plants to various stages of $NO_3^-$ deprivation and then measured uptake in 200 μM $NO_3^-$ over 8 hours. During this time both deprived and non-deprived plants showed changes in uptake rates after 1 hour, and over 7 hours uptake rates more than doubled, even for the non-deprived plants. Hence, uptake characteristics of roots can be expected to change during the course of a depletion experiment. As well as making the interpretation of depletion curves difficult, such changes also mean that the uptake kinetics of the plants at the end of a measurement would not reflect those of the plants at the beginning.

Unfortunately the reason the experiments were as long as they were lay in the form of the *E. nitens* roots. The root systems were generally long (>70 cm), and near the root shoot junction there was a proliferation of lateral roots coming off the main tap root which made up the bulk of the root mass. The tap root itself was two to four times longer than the bulk of the root mass and generally had short laterals along its full length. Fitting these root systems into cuvettes of minimal volume without disturbing the root was difficult and led to high solution to root ratios and the long periods required for depletion. The worst case of which were the roots of the 2.5% RAR plants which were very long (70-90 cm) and slender (Figure 4.13). The problem of timescale was even worse for $NO_3^-$. Based on the low uptake rates of $NO_3^-$ found here it would be difficult to use the depletion method to measure $NO_3^-$ uptake kinetics with similar plants.

Another departure from the preferred depletion protocol of Claassen and Barber (1974) was in the number of points recorded once $C_{min}$ had been reached. Claassen and Barber suggested that for accurate parameter estimation the depletion curves should reach $C_{min}$ and have several points past this point. The 2.5% RAR plants had not reached $C_{min}$, so parameters could not be estimated. Even the 7.5% RAR and 12.5% RAR plants had only just reached $C_{min}$ by the end of the assays. However, this did not affect the calculation of V(C) data, as when further points equal to $C_{min}$ were

extrapolated at regular intervals past the last recorded concentration, there was no effect on the shape of the derived curves.

This experiment was hindered by the problems found using the RAR approach, the main problem possibly being the accumulation of $NO_3^-$ in the culture units. It is assumed that the cause of the accumulation was the unsuitability of the $NH_4^+$:$NO_3^-$ ratio of the nutrient solution. This assumption is based on two main factors. Firstly, all the experiments presented in this chapter have shown uptake rates of $NH_4^+$ to be at least 50% greater than $NO_3^-$ uptake rates. Secondly, RGR did not match the highest RAR, even though there was accumulation of $NO_3^-$. To remedy this problem it would have been necessary at the start of an experiment to adjust the ratio of $NH_4^+$ and $NO_3^-$ to match the ratio in which the two ions were taken up.

Another possible problem with the RAR approach as applied here was that nutrients were added hourly throughout each day with no correction for diurnal changes in uptake. Nutrient uptake shows diurnal variation that closely follows photosynthetic activity (LeBot and Kirkby, 1992; Rideout and Raper, 1994; Scaife and Schloemer, 1994; Delhon et al., 1995, 1996). Hence, continued nutrient additions during darkness, when uptake rates may have been reduced, may have led to nutrient accumulation in the growth units. This problem could be rectified by including diurnal variations in the nutrient addition program.

## *4.5 Conclusions*

The $NH_4^+$ uptake rates measured were generally more than twice those measured for $NO_3^-$. Changing the source of N had no effect on the uptake rates for $NH_4^+$ and $NO_3^-$. In buffered solutions both $NH_4^+$ and $NO_3^-$ uptake rates were higher at pH 4 than at pH 6. Changing pre-treatment or measurement temperatures between 10°C and 20°C did not change the overall preference for $NH_4^+$, and adaptation to reduced root temperatures was rapid (< 24 hrs). Low nutrient status did not change the preference for $NH_4^+$ as an N source for *E. nitens*, and the main response to low nutrient status was to increase root size with respect to shoot size. The difficulties of interpretation of depletion experiments, and the unreliability of the parameter estimates, highlighted the need to utilise an alternative method for quantifying short term (< 1 hr) N uptake by roots.

# 5. Nitrogen and proton fluxes at the surface of *E. nitens* roots

## *5.1 Introduction*

Measurement of uptake via the depletion method yielded useful information about the $NH_4^+$ and $NO_3^-$ uptake characteristics of *E. nitens* roots (Chapter 4). However, the results from the depletion method can be difficult to interpret (Clarkson, 1986; Lee and Clarkson, 1986; Kronzucker et al., 1996) because net uptake is measured without separating the contributions of influx and efflux, and in a depleting solution, efflux could be a quite large component of net uptake (Lee and Drew, 1989; Macduff and Jackson, 1992; Aslam et al., 1996). Another problem with the depletion method is that the period required to reach full depletion (e.g. for a decrease in concentration from 100 μM to ≈ 0 μM) can take up to 10 hours (Table 3.1), during which changes could occur in the uptake characteristics of roots (i.e. uptake regulation).

The MIFE (micro- electrode ion flux estimation) technique is a method of measuring an ion flux at a root surface based on measurements of the concentration gradient for the ions in question. The MIFE technique can yield information about ion fluxes with high spatial (e.g. ≈ 20μm) and temporal resolution (e.g. < 20 sec). High temporal resolution means that it is possible to investigate the dynamics of fluxes. This aspect can be applied to minimise the efflux component of net fluxes to better estimate influx, it also means that measurements can be made in which the immediate effects of treatments can be investigated. High spatial resolution means that localised fluxes can be measured, which is important because fluxes may be different on different parts of the root system. It also means that fluxes can be measured at the surface of just a few epidermal cells and in this way associations between different fluxes can be investigated.

The basis for the MIFE technique is that in solution a gradient in ion activity (equivalent to concentration in dilute solutions) will be established as ions are taken up or excreted by the root. If this ion activity gradient can be measured then fluxes (uptake) can be calculated. The technique has its origins in the vibrating probe work

of Jaffe and Nuccitelli (1974), who used a vibrating, voltage sensitive probe to measure net ionic currents (i.e. the total for all moving ions) around plant roots. With the development of neutral, carrier-based, ion-selective resins it became possible to measure not just the net ionic current but also the current of specific ions (Lucas and Kochian, 1986).

Earliest attempts at estimating ion fluxes with microelectrodes manually mapped ion activity within the unstirred layer around roots, i.e. ion activity was measured at one radial distance from the root and then the electrode was manually repositioned to another distance prior to another measurement (Newman et al., 1987; Ryan et al., 1990; Henriksen et al., 1990, 1992). This approach, although successful, is susceptible to error due to two sources of signal drift. Firstly, liquid ion exchange resin microelectrodes have a small voltage drift associated with their use. Secondly, continued influx or efflux will lead to gradual change in the bulk solution concentration which will also affect the calculation of net flux in a similar manner to that of signal drift. For both these reasons, measurements made at two radial distances at different times will incur some error in the observed differences in activity. If the solution concentration is relatively low and fluxes are high this error is low, otherwise the method is unsatisfactory. A thorough critique of this technique and its problems is presented by Kochian et al. (1992).

A way of avoiding the problems associated with stationary measurements is to vibrate the ion-selective microelectrode between two radial distances at a high enough frequency so that electrode drift is insignificant. Kuhtreiber and Jaffe (1990) first developed this method with a calcium sensitive microelectrode and this was adapted for $Ca^{++}$, $K^{+}$, and, $H^{+}$ fluxes by Kochian et al. (1992).

### 5.1.1 Spatial and temporal variation in fluxes

The eucalypt seedling roots used in the following experiments had a main tap root (length 70-90 mm) with laterals up to 2 mm long. Just below the root shoot junction were the oldest laterals approaching the length of the tap root (Figure 5.6). The 20-50 mm region of the tap root behind the tip was the focus of this study because it was easily accessible and, according to findings with maize and tomato roots, the tap root will have similar but slightly higher uptake characteristics than the

rest of the root surface (Lazof et al., 1992; Zobel et al., 1992). Being away from the growing tip avoided possible interference due to the ion fluxes associated with growth (Ryan et al., 1990; Shabala et al., 1997).

Previous studies using MIFE systems have found high variability in root ion fluxes, both spatially and temporally, in a number of plant species and for a range of ions. For example, fluxes of $K^+$ and $H^+$ in 3-day-old maize roots measured at the same position showed dramatic variation with time (Newman et al., 1987). Another study with maize roots found $K^+$, $Ca^{++}$, and $H^+$ fluxes in 3 day old roots that were constantly changing between influx and efflux (Ryan et al., 1990). Ammonium and $NO_3^-$ fluxes in 7 day old barley roots varied between different measurement positions along a root but these trends changed with time (Henriksen et al., 1990, 1992). Nitrate fluxes in 2 week and older tomato roots showed trends in distance from the root tip of the tap root and between primary and secondary roots but these results were also highly variable (Zobel et al., 1992).

These results suggested that to characterise $NH_4^+$ and $NO_3^-$ uptake by *E. nitens* roots using the MIFE system it was first necessary to quantify the spatial and temporal variation in fluxes, i.e. the repeatability of the measurements. The amount of spatial and temporal variation was also important for the design of experiments investigating treatments effects because treatment effects would need to be distinguishable from inherent spatial and temporal variability.

### 5.1.2 Proton fluxes associated with N fluxes

The MIFE system offered the potential to simultaneously measure $NH_4^+$, $NO_3^-$, and $H^+$ fluxes and thereby investigate the mechanisms of $NH_4^+$ and $NO_3^-$ uptake because both $NH_4^+$ uptake and $NO_3^-$ uptake are strongly linked to $H^+$ fluxes (Breteler, 1973; Schlee and Komor, 1986; McClure et al., 1990; Knoepp et al., 1993). At least at low concentrations, $NH_4^+$ and $NO_3^-$ uptake into roots could be via symports with $H^+$. Strong evidence for this link has been put forward for $NO_3^-$ (Glass et al., 1992; Meharg and Blatt, 1995), but there is still much deliberation over whether $NH_4^+$ uptake is active via a symport or specific ATPase, or whether it is passive via a uniport (Walker et al., 1979a, 1979b; Kleiner, 1981; Ullrich, 1992; Wang et al., 1994). If uptake of both $NH_4^+$ and $NO_3^-$ is via $H^+$ symports then there should be an association between the observed fluxes of nitrogen and $H^+$.

Assimilation of $NH_4^+$ and $NO_3^-$ is also linked to $H^+$ fluxes. Ammonium assimilation within the root produces at least one $H^+$, which must be removed from the root to maintain pH (Raven and Smith, 1976). Nitrate assimilation, whether in the root or shoot, produces the equivalent of about one hydroxide ion which also must eventually be removed via the root (Raven and Smith, 1976). As the MIFE system could measure $NH_4^+$, $NO_3^-$, and $H^+$ fluxes simultaneously over a very small area of the root surface (equivalent to a number of cells) it offered the opportunity to observe the interactions between these fluxes with high temporal resolution. The stoichiometry of these fluxes would reflect something about the nature of the underlying uptake and assimilation processes.

### 5.1.3 pH

As outlined in Chapter 4, there are varied responses of $NH_4^+$ and $NO_3^-$ uptake to changes in substrate pH. The effect of pH on long term uptake (1-7 hours) of $NH_4^+$ and $NO_3^-$ was investigated in Chapter 4 using the depletion method, and both $NH_4^+$ and $NO_3^-$ uptake were higher at pH 4 than pH 6. A problem with measuring uptake at different pH is the difficulty in maintaining stable pH throughout the term of the measurement. The pH must be stabilised using either constant pH adjustment (Bloom, 1989), or, as in Chapter 4, by using buffers. Constant pH adjustment is difficult and buffers can have their own physiological effects (Medeiros et al., 1993; Nicholas and Harper, 1993).

The MIFE system provided the possibility of measuring the effects of pH on uptake of $NH_4^+$ and $NO_3^-$ on such a small timescale that it avoided the need for pH adjustment. Measuring $H^+$ fluxes at the same time also made it possible to determine the effect of external pH changes had on pH regulation by the root.

### 5.1.4 Ammonium inhibition of nitrate uptake

As discussed in Chapter 4, the presence of $NH_4^+$ can inhibit the uptake of $NO_3^-$. However, in Chapter 4, no evidence was found for such inhibition in *E. nitens* roots. The MIFE system offered an alternative means of investigating the possible interactions between $NH_4^+$ and $NO_3^-$ uptake in *E. nitens* roots.

### 5.1.5 Kinetics

Kinetic parameters were estimated by the depletion method in Chapter 4, but this method has received various criticisms (Wild et al., 1979; Mullins and Edwards, 1988). The MIFE technique offered an alternative method for estimating uptake kinetics. For example, MIFE systems had already been successfully used to measure the concentration dependence of $K^+$ and $NO_3^-$ uptake in maize (Newman et al., 1987; McClure et al., 1990). There are two main advantages of the MIFE system for measuring kinetics, firstly, concentration changes and measurements can be made over relatively short periods (compared with other methods) so efflux can be minimised, and secondly, the actual concentration at the root surface is known instead of the bulk solution concentration which, even with mixing of the solution, can be considerably higher or lower than the concentration at the root surface (Newman et al., 1987).

### 5.1.6 Excision

The use of excised roots in radioactive tracer uptake experiments has been the basis for much of our current knowledge of nutrient uptake (Huang et al., 1992). For nutrients where there is a radioisotope available, short term excised root experiments have been a very important method for investigating nutrient uptake by roots. Recently, the uptake activity of excised roots of field grown plants have been used both to investigate responses to localised fertilisation (Jackson and Caldwell, 1991; Bassirirad et al., 1993) and in a method aimed at identifying nutrient deficiencies in the field (Jones et al., 1991). Wide usage of excised roots continues despite evidence that excision and general physical disturbance involved in excised root experiments have detrimental effects on root processes (Glass, 1978; Gronewald and Hanson, 1980; Bloom and Caldwell, 1988, Bloom and Sukrapanna, 1990). The MIFE technique offered an hitherto unexplored opportunity to investigate the effects of shoot excision on root processes, and help decide to what extend the usage of excised roots could be justified.

### 5.1.7 Objectives

The aim of this chapter was to answer the following questions regarding $NH_4^+$ and $NO_3^-$ uptake by *E. nitens* roots using the MIFE technique:

- What was the extent of the spatial and temporal variation of $NH_4^+$ and $NO_3^-$ fluxes along the tap root of *E. nitens* seedlings?
- What was the degree of synchrony that existed between $NH_4^+$, $NO_3^-$, and $H^+$ fluxes?
- To what extent were $NH_4^+$, $NO_3^-$, and $H^+$ fluxes effected by changes in pH between pH 4 and pH 6?
- Did the presence of $NH_4^+$ inhibit the uptake of $NO_3^-$, and what is the nature of this inhibition?
- What were the kinetics of $NH_4^+$ and $NO_3^-$ fluxes and did these differ from those estimated via the depletion method and for other species?
- What effect did root excision have on root fluxes and what is the timescale of any effect?
- What advantages and disadvantages did the MIFE technique exhibit for studies of $NH_4^+$ and $NO_3^-$ uptake?

## *5.2 Methods*

### 5.2.1 MIFE Theory

In the MIFE method, activity of ions are measured at two positions in the solution surrounding the root. These activities and Fick's first law of diffusion are used to calculate ion fluxes (Henriksen et al., 1992):

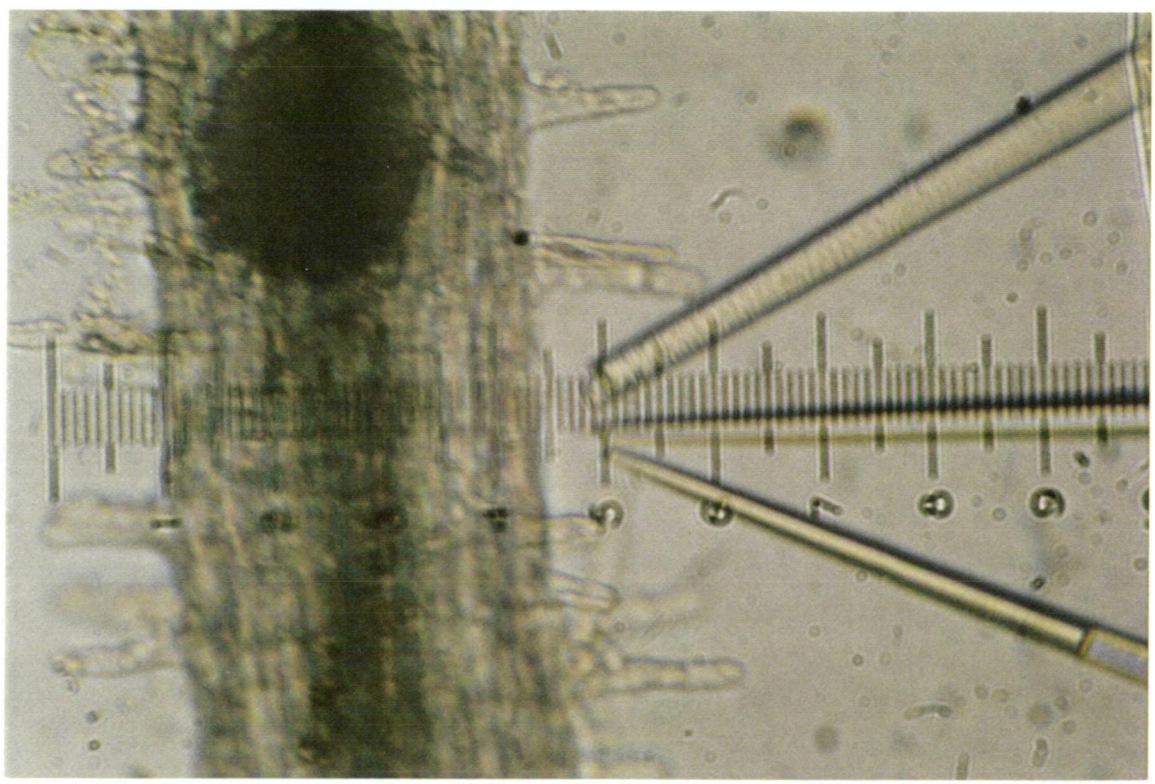
$$J_x = \frac{KD_x(C_2 - C_1)}{r\ln(R_2/R_1)} \qquad \textit{Equation 5.1}$$

where $J_x$ is the net flux of ion x per unit root area per unit time (nmol $m^{-2}$ $s^{-1}$), K is a units conversion coefficient to express $J_x$ in units of nmol $m^{-2}$ $s^{-1}$, $D_x$ is the self diffusion coefficient for ion x ($cm^{-2}$ $sec^{-1}$), $C_1$ and $C_2$ are the ion activities (concentrations) at positions 1 and 2 (nmol $cm^{-3}$), r is the radius of the root (μm), and $R_1$ and $R_2$ are the radii of the two measurement positions (μm). The self diffusion coefficients used were ($cm^2$ $s^{-1}$); $NH_4^+$, $1.95\times10^{-5}$; $NO_3^-$, $1.90\times10^{-5}$; $H^+$, $9.17\times10^{-5}$ (Robinson and Stokes, 1959).

Equation 5.1 assumes that ion gradients around roots can be described by cylindrical diffusion which is appropriate for small roots such as those of eucalypt seedlings (Henriksen et al., 1992). The effect of bulk flow of solution to the root due to transpiration is assumed to be negligible based on the work of Henriksen et al. (1992).

During flux measurements, the chamber was oscillating such that the electrodes moved at 5 s intervals between two positions ($R_1$ and $R_2$) close to the surface of the root (Figure 5.1). Flux estimates are based on a running average of concentration differences between these two points. Oscillation ensures that any drift in electrode response would be reflected in the concentrations at both distances and thereby minimise the effects of electrode drift on flux estimates (see Section 5.2.3).

***Figure 5.1.*** *Eucalypt root and ion selective microelectrodes during a flux measurement. The electrodes are at the closest point of the oscillation. During measurement the electrodes were oscillated between this point and a position 50 μm further from the root surface. Each minor graticule is the equivalent of 5.5 μm.*



### 5.2.2 MIFE Apparatus

The MIFE system consisted of several parts: a microscope holding the electrodes and plant chamber, stepping motor and controller, a pre-amplifier and

amplifier, and a digital/analogue converter, which in turn was connected to a personal computer containing the data acquisition and storage software.

The apparatus was built around a compound microscope (Leitz Wetzlar) mounted at 90° to the horizontal. A chamber holding the plant in solution was mounted upright on the stage so that the horizontal root was in the microscopes line of focus. The measurement chamber (Figure 5.2.) allowed seedlings to be positioned so that the tap root was separated from the secondary roots and easily accessible from above. The tap root was gently manoeuvred into grooves in perspex bridges and then gently held in place with pieces of nylon tubing positioned from above. The chambers were fabricated from acrylic sheeting fastened with flowable silicon sealant (DowCorning, RTV 734). The solution volume of the chamber was 12 mL. Solution was supplied to the chamber at a flow rate of 10 mL $min^{-1}$ using a peristaltic pump. It took approximately 4 minutes to completely change the solution. The shoot was illuminated using an optical fibre light source directed in such a way as not to illuminate the roots. The photon flux density at the leaf surface was approximately 200 $\mu mol\ m^{-2}\ s^{-1}$.

The microscope stage containing the chamber could be moved in three dimensions under the control of an hydraulic micro-manipulator (Narashige WR-88). The vertical movement controller of the stage was connected to a computer controlled stepper motor (Superior Electric MO61-CE08) that could vibrate the stage in an up and down movement with both frequency and amplitude under the control of the data acquisition software. Hence, in contrast to other published systems the one used here moved the plant root with respect to the electrodes as opposed to the electrodes moving with respect to the plant. The root movement (40 μm) was not thought to affect root activity as the disturbance was much less than would occur if the solution was aerated. The three ion-selective microelectrodes were mounted vertically above the plant chamber in a modified, adjustable, 3-electrode holder (Narashige). A KCl/Agar reference electrode was placed in the solution of the chamber at the end furthest from the ion selective electrodes. The experimental chamber is shown in Figure 5.3.

***Figure 5.2.*** *The experimental chamber used for flux measurements. During measurements the chamber was oscillated vertically whilst the electrodes were kept stationary.*

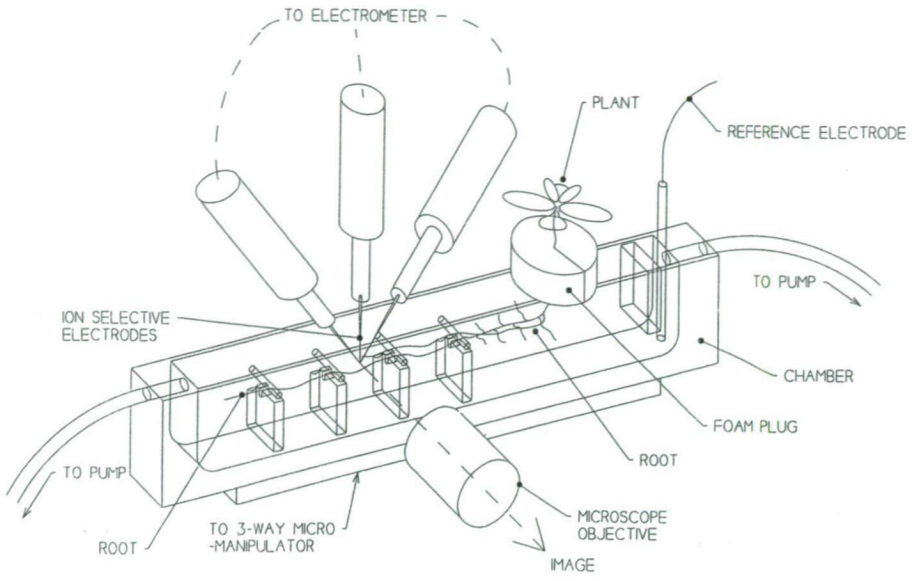
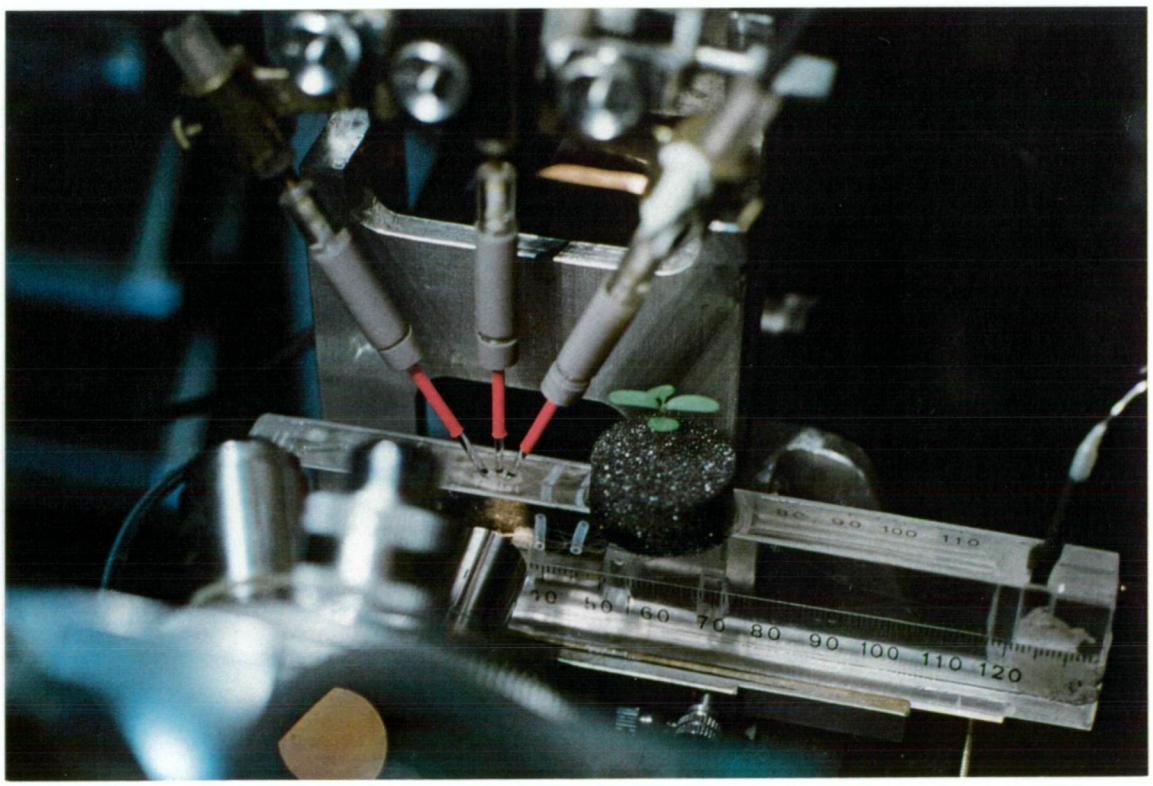
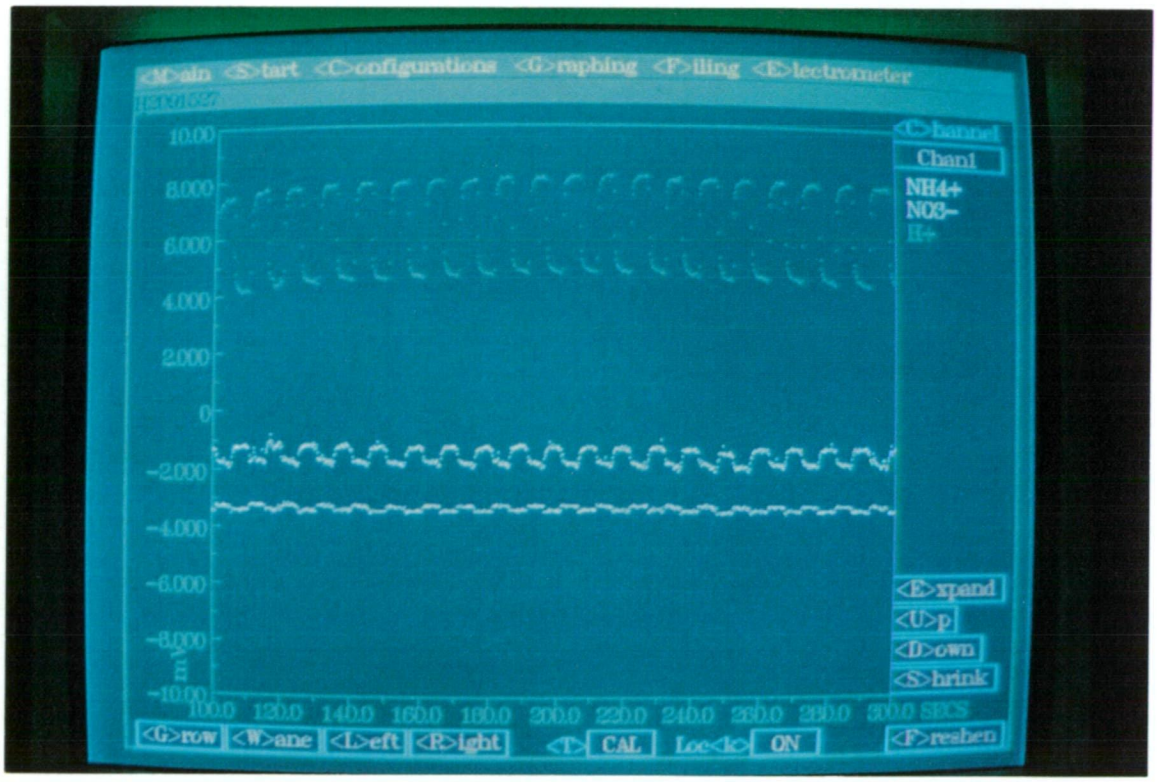



***Figure 5.3****. A plant in the experimental chamber during a flux measurement.*



The voltage output from the electrodes was digitised using an analogue-to-digital interface card (LA-200 Digiflow Pty Ltd) in an IBM-compatible PC. The LA-200 card also controlled the stepper motor and was used to adjust the offsets on the 4 channel electrometer. The interface was controlled by a custom made software package, CHART, which also collected and stored the digitised output. When measuring, the electrodes were oscillated at 0.1 Hz between 50 and 90 μm from the root surface (via moving the roots). The output from the electrodes produced a trace, an example of which is presented in Figure 5.4.

***Figure 5.4.*** *Voltage output from the electrodes during flux measurements as they appear in the CHART program. The different traces represent different electrodes ($NH_4^+$, $NO_3^-$, and $H^+$).*



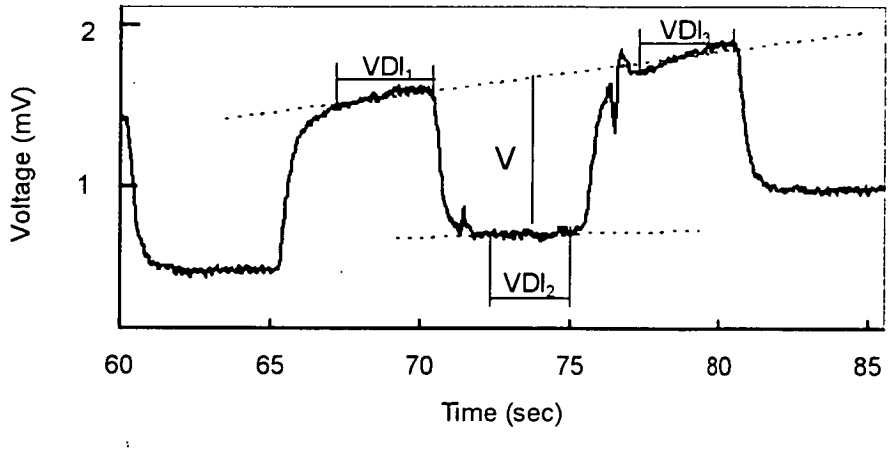

### 5.2.3 Flux calculations

The stored digitised output from the oscillating electrodes was converted to flux values by the process described in Shabala et al. (1997). A detailed view of the output from a single electrode is shown in Figure 5.5, for which, at t = 70 the electrode was rapidly moved from a position 90 μm from the root surface to a position 50μm from the root surface. Concurrently, the electrochemical potential decreased and, after a slight delay, stabilised. At t = 75 s the electrode moved back to 90 μm and the

electrochemical potential increased and, again after a short delay, stabilised. These cycles were repeated with a period of 10 s.

To calculate flux, the voltage difference between the two positions (in this case 50 μm and 90 μm) must be calculated from the voltage trace. In doing this some of the trace is discarded. Valid data intervals (VDI) are made up of the stable voltage readings measured at each position, excluding that part of the trace associated with changing position and stabilisation. In this case, the first two seconds between position changes was discarded. The voltage difference was calculated between three consecutive VDIs ($VDI_1$, $VDI_2$, and $VDI_3$ in Figure 5.5). A line was drawn between the mean values of $VDI_1$ and $VDI_3$ and the voltage difference between the two positions was calculated as the mean distance between this line and the mean of $VDI_2$. The time corresponding to this difference was regarded as the midpoint of $VDI_2$. The flux 5 seconds later used $VDI_3$ as $VDI_1$ and the difference calculated on this next triplet with this process repeated along the trace. The voltage difference between the two positions was then used in Equation 5.1 to calculate fluxes.

***Figure 5.5.*** *Example of voltage output from an ion selective microelectrode. The flux estimate was based on a running average of the concentration difference between the two different distances. The gradual increasing voltage reading can be due to either gradual concentration change or electrode drift. The output during the position changes are not used in flux estimation. (Adapted from Shabala et al., 1997).*




### 5.2.4 Electrodes

Electrodes were made from 1.5 mm (external diameter) borosilicate blanks (Clark Electromedical GC 150-10). The blanks were pulled to < 1μm diameter tips

using a vertical pipette puller. Electrodes were baked at 200 °C for 4 hours after which they were silanized by adding a single drop of tributylchlorosilane (Fluka 90796) to a closed container with the blanks. After 10 minutes the cover was removed and the electrodes baked for a further 30 minutes. The shanks of the silanized blanks were shortened to approximately 3 cm length and the shortened end flamed to remove rough edges. Blanks were mounted horizontally on a micro-manipulator under a stereo microscope. The <1 μm diameter tip was broken back to 2-3 μm diameter by gently moving it into a flat glass surface. Satisfactory blanks were back filled. The backfilling solutions were: for $NH_4^+$, 0.5 M $NH_4Cl$; for $NO_3^-$, 0.5 M $KNO_3$ + 0.1 M KCl; and for $H^+$, 15 mM NaCl + 40 mM $KH_2PO_4$ adjusted to pH 6 with NaOH. Immediately after backfilling the electrodes were front filled with their respective resins to a column length of approximately 200 μm ($NH_4^+$, Fluka 09882; $NO_3^-$, Orion 219568-A01; $H^+$, Fluka 95291). After filling, and before use, the electrodes were left to equilibrate in either 0.5 M $NH_4NO_3$ + 0.1 M KCl for $NH_4^+$ and $NO_3^-$ electrodes or 0.1 mM $CaCl_2$, 0.1 mM KCl adjusted to pH 6 with NaOH for $H^+$ microelectrodes. The drift of the electrodes was generally less than 0.2 mV $hr^{-1}$ and the electrodes were stable for as long as 48 hours. The resistance of the electrodes was ≈2 GΩ.

Suitable electrodes were fitted to the electrode holder and then calibrated. The $NH_4^+$ and $NO_3^-$ electrodes were calibrated in 25, 50,100, and 1000 μM $NH_4NO_3$ + 200 μM $CaSO_4$. The $H^+$ electrodes were calibrated in pH 7, 6, and 4.5 solutions made up with phosphate buffer containing 100 μM $CaCl_2$ and 100 μM KCl.

A Ag/AgCl reference electrode was used consisting of a chlorided silver wire inserted into a length of 2 mm diameter nylon tubing filled with 1 M KCl in 2% agar. In methodological experiments, $K^-$ leakage from the reference electrode led to no more than 3 μM $K^+$ in the measurement region (Shabala pers. comm.).

### 5.2.5 Plant culture

Seeds of *E. nitens* (source as described in Chapter 4.2.1) were surface sterilised by soaking in NaClO (1% Cl) for 15 minutes and then rinsed thoroughly in DI water. Washed seeds were then evenly spread over two layers of filter paper in the base of a 9 cm diameter petri dish prior to the lid being replaced. Seeds in petri dishes were

kept moist and in darkness at 20°C. When the stems of the plants were 20 mm in length (approximately 10 days) the seedlings were placed in foam collars (see Chapter 4.2.1) and transferred to culture units.

Culture units consisted of 5 L polypropylene tubs (120 mm x 300 mm x 160 mm) with grey PVC lids. The container lids had 23 x 20 mm diameter holes into which foam collars containing plants were placed. Silicon tubing and 17 gauge hypodermic needles were used to aerate the solution, and light was excluded from the roots with black polythene sheeting. Plants were grown under 400 W metal halide lamps, that produce a photosynthetic photon flux density at the leaf surface of 600 $\mu mol\ m^{-2}\ sec^{-1}$. The nutrient solution was diluted Ingestad solution, the composition of which was described in Chapter 4.2.1. The $NH_4^+$ and $NO_3^-$ concentrations of the medium were 80 μM and 120 μM, respectively. The nutrient solution was changed every 4 days. The pH of the nutrient solution was 6.2 ± 0.4. After approximately 17 days the plants were used for flux measurements. At this stage the plants had 2 sets of true leaves and tap roots were approximately 80 mm long.

The day prior to a flux measurement, 4 plants were removed from the culture unit, a clean foam collar fitted, and the plants placed in a pre-treatment container under light. The pre-treatment container was a 2 litre polypropylene tub with a grey PVC lid. The solution was aerated with silicon tubing and 17 gauge hypodermic needles, and light was excluded from the roots with black polythene sheeting. The pre-treatment solution contained 100 μM $NH_4NO_3$ and 200 μM $CaSO_4$ (pH 6.1)

### 5.2.6 Flux measurement

After overnight pre-treatment, plants were positioned in measurement chambers and the measurement chamber placed back under lights in the culture room with roots shielded from light and the solution aerated. The plants were left in this way for a minimum of 1 hour, during which the solution was refreshed every 15 minutes.

Prior to flux measurements, plants were placed on the microscope stage with the root tip in the field of vision and the rate of tip elongation measured over 15 minutes. During this time, solution was circulated through the chamber. Elongation rates were generally of the order of 150 $\mu m\ hr^{-1}$. Tip growth was slightly gravitropic but this was insignificant at the timescale of the experiments.

The basic measurement solution was the same as the pre-treatment solution, 100 μM $NH_4NO_3$ and 200 μM $CaSO_4$ (pH 6.1). In some previous MIFE-type experiments in which N fluxes were measured, a high concentration of $MgSO_4$ (1.1 mM) was used to provide uniform ionic strength for measurements (Henriksen et al., 1990, 1992; Henriksen and Spanswick, 1993). However, preliminary experiments with *E. nitens* indicated that root elongation halted in a medium containing 1.1 mM $MgSO_4$. These plants had been grown in low ionic strength solution (conductivity 25 μS) then moved to a solution containing 1.1 mM $MgSO_4$ (250 μS). Based on the evidence that ionic shock can significantly affect root function (Bloom and Epstein, 1984; Bloom and Finazzo, 1986) it was possible that the presence of 1.1 mM $MgSO_4$ may have been proving deleterious to *E. nitens* root growth. In the concentration ranges used in the experiments in this chapter ionic strength had been found to have little effect on electrode characteristics (Henriksen et al., 1990; Kochian et al., 1992). The removal of 1.1 mM $MgSO_4$ from the measuring medium resulted in measurable elongation rates.

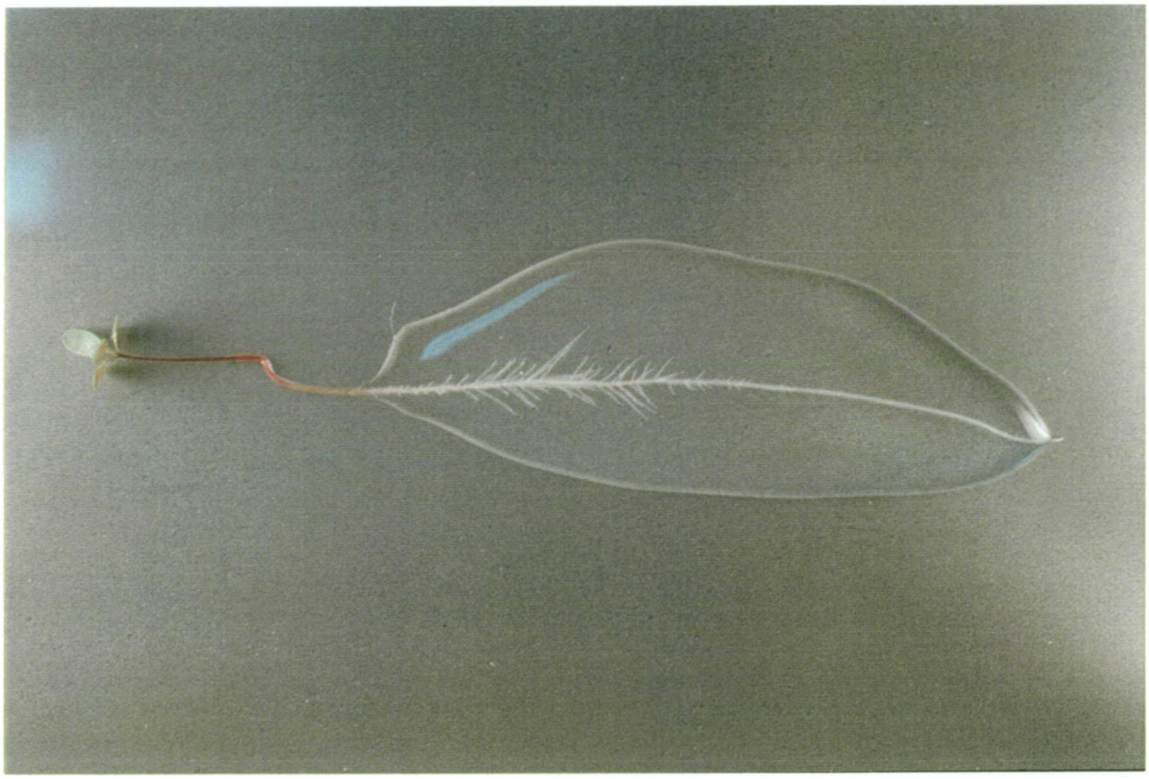
After the root length measurements, plants were ready for flux measurements. Solution flow was stopped and the electrodes positioned 90 μm from the root surface by manipulating both the chamber and the electrodes. With electrodes in position the measuring program commenced. Electrodes were oscillated between 90 and 50 μm with a frequency of 0.1 Hz. A flux measurement was generally 4 minutes of stable flux readings after which time the electrode was moved to another position on the root or the solution concentration was changed. At each measurement, the position was noted by both the horizontal micrometer reading of the micro-manipulator, and the physical characteristics of the root with respect to the electrodes. Using these two factors each measurement position could be returned to with a high level of accuracy (within 5 μm). At each measurement position a photograph was taken through the microscope lens using an Olympus OM-2 SLR camera with a T-mount microscope adapter.

### 5.2.7 Eucalypt root morphology

Eucalypt roots are much slower growing than roots of model plants like maize or barley, (typical elongation rates of 150 μm $hr^{-1}$ compared to 1500 μm $hr^{-1}$ for maize). Eucalypt roots are also much smaller (the diameter of the roots used was approximately 220 μm compared to approximately 1000 μm for maize). At the time

of measurements the eucalypt tap roots were approximately 80 mm in length. At this stage, secondary roots up to 60 mm in length extended from the region 5 to 15 mm from the root/shoot junction. Within the measurement region of the tap root, 20-50 mm from the root tip, there were already laterals developing up to 3 mm in length. Root hairs were present to varying densities in the zone of measurement. The length of root hairs ranged from 50-150 μm long but were generally 50 μM long. A typical plant is shown in Figure 5.6.

*Figure 5.6. A eucalypt root similar to those used in MIFE measurements. The length of the root system is approximately 85 mm.*



### 5.2.8 Spatial and temporal variation in fluxes

Fluxes were measured at 7 positions along each root within the region 20-50 mm from the root tip. At each position, fluxes were measured for at least 4 minutes. Every 15 minutes the pump was turned on for 1 minute to refresh the solution. The measurement solution was the same as the pre-treatment solution (100 μM $NH_4NO_3$, 200 μM $CaSO_4$, unbuffered pH = 6.1). The 7 positions were approximately evenly spread within the 20-50 mm region from the root tip. Actual positions were determined by the accessibility for the electrodes. After an initial set of measurements,

each position was re-measured at random. Both sets of measurements were completed in approximately 90 minutes. This process was carried out on three roots.

For statistical analysis the positions were given a nominal value of 1-7 in terms of there distance from the tip. The temporal variability was first quantified using scatterplots and pairwise t-tests. For further analysis, the mean of the two times at each position on each root was used as the flux value at that position on that root.

The differences between fluxes for individual ions measured at each position were quantified by using a randomised complete block design ANOVA using roots treated as blocks. Differences between $NH_4^+$, $NO_3^-$, and $H^+$ fluxes were determined using a completely randomised design with fluxes pooled from roots and different root positions. Pairwise comparisons between means were determined by least significant differences ($P = 0.05$). The relationship between $NH_4^+$, $NO_3^-$, and $H^+$ fluxes was investigated using scatterplots and correlation analysis.

### 5.2.9 pH

Plants were set up in the normal manner (Section 5.2.5), but in this case the pre-treatment was 100 μM $NH_4NO_3$ and 200 μM $CaSO_4$ adjusted to pH 6 with 0.5 M $H_2SO_4$. After measuring tip growth, an initial flux measurement was made at three positions and then the solution changed to 100μM $NH_4NO_3$ and 200μM $CaSO_4$ adjusted to pH 4 with 0.5 M $H_2SO_4$. The fresh solution was flowed through the chamber for 10 minutes and then the plant in the chamber was removed to the culture room for an hour during which the solution was refreshed every 15 minutes. After 1 hour, fluxes were re-measured at the same three positions in pH 4 solution and then the solution changed back to pH 6, left for another hour, and fluxes re-measured at the same three positions in pH 6 solution.

Fluxes at each position where pooled to give an average flux and differences between fluxes at the three different times (pH 6 prior, pH 4, pH 6 post) were analysed using a randomised complete block design ANOVA. Pairwise comparisons between means were determined by least significant differences ($P = 0.05$).

### 5.2.10 Ammonium inhibition of nitrate uptake

Plants were set-up in the normal manner (Section 5.2.5) in 100 μM $NH_4NO_3$, 200 μM $CaSO_4$ and $NH_4^+$, $NO_3^-$, and $H^+$ fluxes measured at three positions. At the end of these initial measurements, the solution was changed to 50 μM $Ca(NO_3)_2$ plus 200 μM $CaSO_4$ and this new solution circulated for 10 minutes. After the solution change the plants in the chambers were removed to the growth room under lights for one hour with solutions continuously aerated and replaced every 15 minutes. After 1 hour, plants were returned to the MIFE system and $NH_4^+$, $NO_3^-$, and $H^+$ fluxes re-measured at the same three positions. At the end of this flux measurement the solution was changed back to the original solution of 100 μM $NH_4NO_3$, 200 μM $CaSO_4$ and fluxes re-measured.

The fluxes measured at each of the three positions where pooled to give a mean flux. Differences between fluxes in each solution for $NH_4^+$, $NO_3^-$ and $H^+$ were determined using a randomised complete block design ANOVA. Pairwise comparisons between means were determined by least significant differences ($P$ = 0.05).

### 5.2.11 Kinetics

Plants were set up in the normal manner (Section 5.2.5) and an initial flux measurement made in the pre-treatment solution (100 μM $NH_4NO_3$ and 200 μM $CaSO_4$). After the initial measurement, the $NH_4NO_3$ concentration was reduced to 25 μM and circulated for 10 minutes. Recirculation was then stopped, fluxes measured, and then the solution was changed again. Apart from the initial solution change to 25 μM, further concentrations changes took 4 minutes. Fluxes were measured in this way at 25, 50, 100, 150, 250, 500, and 1000 μM. The total time for this series of measurements was approximately 80 minutes.

For each root only one position was measured at all the concentrations. This was both to minimise time between measurements and to minimise any small effects of repositioning between measurements. Once initially positioned the electrodes where not repositioned between concentration changes or measurements.

Due to the flux measurements being measured in static as opposed to stirred solutions the concentration at the root surface is not that of the bulk solution. Root

surface concentrations were estimated from those measured at 50 and 90 μm by rearranging Equation 5.1 to solve for C at a radius equal to that of the root radius:

$$C_0 = C_1 - \frac{J_x r \ln(R_1/r)}{KDx} \qquad \text{Equation. 5.2}$$

where $C_0$ is the concentration at the root surface, $C_1$ is the concentration 50 μm from the root surface, $J_x$ is the ion flux, $R_1$ is the radius of the closest electrode position, r is the radius of the root, $D_x$ is the self diffusion coefficient for the ion in question, and K is a units conversion coefficient to express $J_x$ in units of nmol $m^{-2}$ $s^{-1}$. Estimates of root surface concentrations were made for each bulk solution concentration for each plant. These values were generally 1% less than the concentrations at 50 μM from the root surface. The assumptions made here were rather simplistic in that they do not take into account the ion exchange capacity of the root surface (Walker and Pitman, 1976).

The estimated root surface concentrations were used to plot the concentration dependence of uptake, which were in turn used for the estimation of kinetic parameters $K_m$ and $V_{max}$. To estimate the kinetic parameters the pooled concentration uptake curve was fitted to the Michaelis-Menten formula [$V = (V_{max}C) / (K_m + C)$] using the non-linear curve fitting function of the computer package Sigmaplot (Jandel Scientific). In the equation V is the net influx (nmol $m^{-2}$ $sec^{-1}$), $V_{max}$ is the maximum influx, and $K_m$ is the concentration (μM) at which net influx is half of $V_{max}$.

### 5.2.12 Excision

Plants were set-up in the normal manner (5.2.5) in 100 μM $NH_4NO_3$, 200 μM $CaSO_4$ and $NH_4^+$, $NO_3^-$, and $H^+$ fluxes measured at three positions. Plants in the chambers were then removed to the growth room under lights for one hour during which solutions were continuously aerated and replaced every 15 minutes. After 1 hour, plants were returned to the MIFE system and $NH_4^+$, $NO_3^-$, and $H^+$ fluxes re-measured at the same three positions. At the end of the flux measurement the shoot was excised just above the root/shoot interface without disturbing the root. After this, the plants were returned once again to the growth room. After 40 minutes plants were returned to the MIFE system and $NH_4^+$, $NO_3^-$, and $H^+$ fluxes re-measured at the same

three positions. Fluxes were re-measured twice at hour intervals after this first post-excision measurement.

### 5.2.13 Statistical analysis

Statistical analyses were carried out using the computer package SAS (SAS Institute). The SAS procedure ANOVA was used for analysis of variance.

## *5.3 Results*

### 5.3.1 Spatial and temporal variation in fluxes

Axial scans of three roots showed relatively uniform fluxes of $NH_4^+$, $NO_3^-$, and $H^+$ (Figure 5.7). There was some temporal variability found between repeat measurements at each position and this is shown with more clarity in scatterplots of the first measurement against the second (Figure 5.8). Most temporal variation was with $NH_4^+$ fluxes, which were on average almost 20% less in the second measurement. A pairwise t-test showed that the $NH_4^+$ fluxes for the second measurement were significantly less than the first measurement ($P << 0.01$). The same test for $NO_3^-$ and $H^+$ fluxes found no significant difference between fluxes measured at different times ($NO_3^-$, $P = 0.10$; $H^+$, $P = 0.58$). This result agrees with preliminary measurements in which fluxes varied little when continuously measured at one position for up to 2 hours.

***Figure 5.7.*** *Fluxes of $NH_4^+$, $NO_3^-$, and $H^+$ within the region 20 to 50 mm from the root tip. Graphs a, b, and c represent 3 different roots with 7 different positions measured on each root. Position 1 is closest to the root tip. Open and closed symbols represent the two different times at which the flux was measured at each position.*

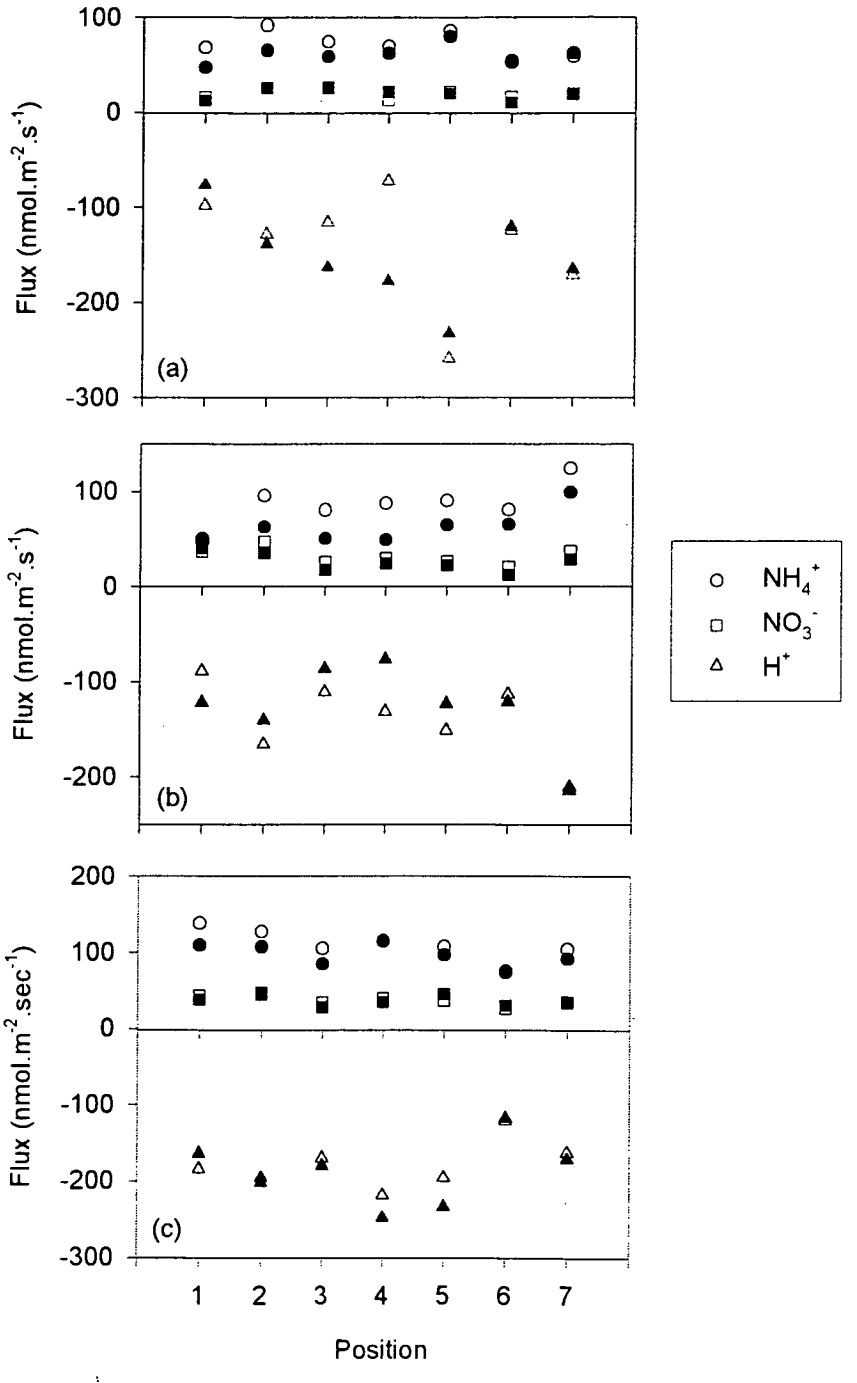
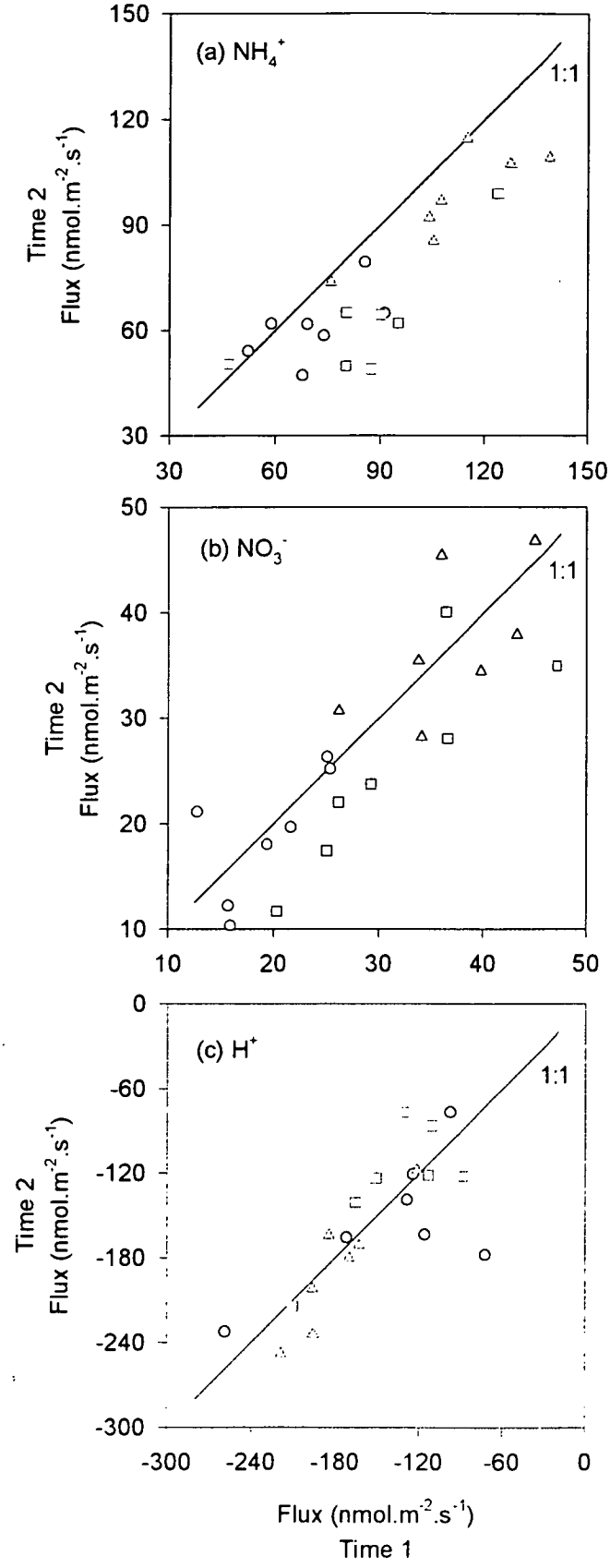


***Figure 5.8.*** *Scatterplots of flux measurements at different times for each position of each root, (a) $NH_4^+$, (b) $NO_3^-$, and (c) $H^+$. Different symbols represent 3 different plants. The line is 1:1.*



For the analysis of flux differences between positions, the results at the two measurement times for each position were pooled. As with the temporal variation between measurement times, spatial variation in fluxes along roots was also small (Figure 5.7). Based on the results of the randomised complete block design ANOVA there were significant differences between roots for both $NH_4^+$ and $NO_3^-$ fluxes ($P < 0.01$) but the differences between roots for $H^+$ fluxes were not significant ($P = 0.06$). When the differences between roots are taken into account there was no difference between measurement positions for the $NH_4^+$ ($P = 0.51$) and $H^+$ ($P = 0.16$) fluxes measured at seven different positions. There was a slight but significant difference between measurement positions for $NO_3^-$ ($P = 0.04$) fluxes but this was only between the extremes of the measured range of fluxes.

Although there were significant differences between ion fluxes measured on different roots these differences were much less than overall differences between the three different ions. The pooled means for the different fluxes measured on all three roots were, for $NH_4^+$, 82 nmol $m^{-2}$ $s^{-1}$, for $NO_3^-$, 28 nmol $m^{-2}$ $s^{-1}$, and for $H^+$, -154 nmol $m^{-2}$ $s^{-1}$. The difference between these three values was highly significant ($P << 0.01$). Hence, the influx of $NH_4^+$ was about 3 times higher than that of $NO_3^-$, and there was a large efflux of $H^+$.

The heterogeneous morphology of the root structure is hard to quantify, but the general trend was that the apical end of the measurement range had no laterals whilst the distal region had well developed laterals. In all of the experiments neither root hair density nor the presence of lateral roots had any obvious effect on the magnitude of fluxes.

As evident in Figure 5.9 there was a consistent relationship between the fluxes measured simultaneously at each position. There were strong correlations between the fluxes of $NH_4^+$ and $NO_3^-$ ($r = 0.67$), and $NH_4^+$ and $H^+$ ($r = -0.67$), whereas the correlation between $NO_3^-$ and $H^-$ was not as strong ($r = -0.49$) (Pearson's correlation coefficient). The ratios of $NH_4^+$ and $H^+$ to that of $NO_3^-$ for the different measurements are shown in Table 5.1.

***Figure 5.9.*** *The relationship between different fluxes measured simultaneously. The data in the scatterplots is the pooled data at each of 7 positions measured on two different occasions on each of 3 roots.*

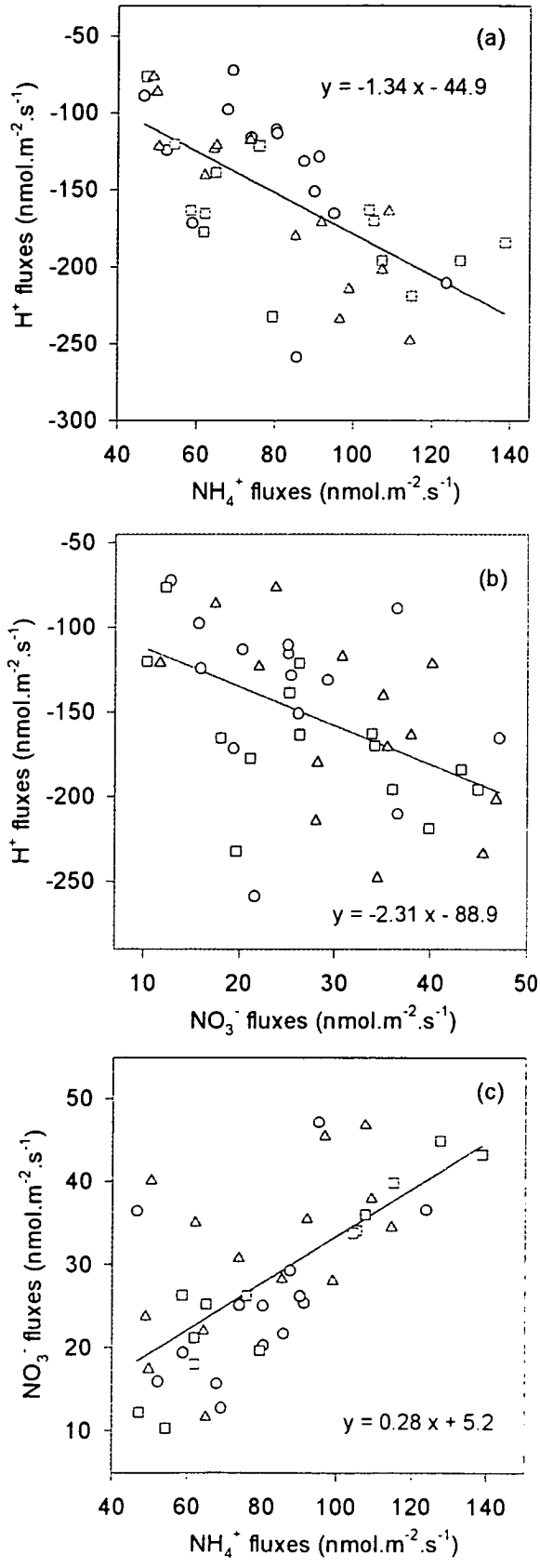


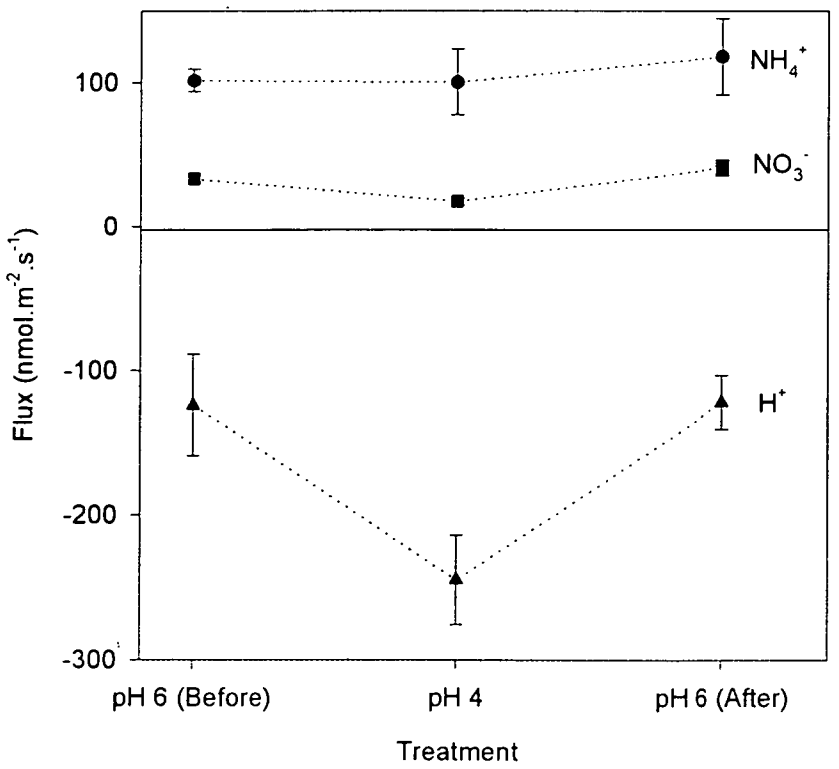
*Table 5.1. The average ratios of ion fluxes for 42 individual measurements. The values are means (± SEM, n=42) of the proportion of the $NH_4^+$ and $H^+$ fluxes with respect to the $NO_3^-$ flux.*

| $NH_4^+$ | $NO_3^-$ | $H^+$ |
|---|---|---|
| 3.11 (± 0.14) | 1 | -5.96 (± 0.36) |

### 5.3.2 pH

The reduction of pH from pH 6 to pH 4 significantly decreased net $NO_3^-$ influx ($P$ = 0.003) and significantly increased net $H^+$ efflux ($P$ = 0.02), but did not affect $NH_4^+$ fluxes (Figure 5.10). The flux of $NO_3^-$ was halved at pH 4 compared to that at pH 6, whereas, $H^+$ efflux was doubled with this pH change.

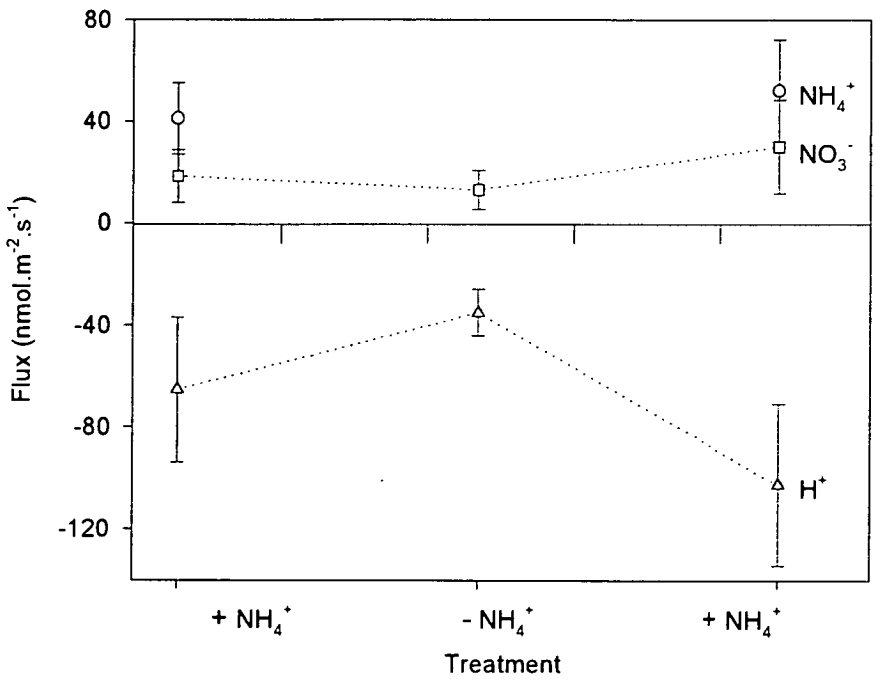
*Figure 5.10. Effects of pH on $NH_4^+$, $NO_3^-$, and $H^+$ fluxes. Fluxes are the means of 4 plants (± SEM). Measurement solution was 100 µM $NH_4NO_3$ plus 200 µM $CaSO_4$ adjusted to pH 6 or pH 4 with $H_2SO_4$. Roots were equilibrated at each pH for 1 hour prior to measurements.*



### 5.3.3 Ammonium inhibition of nitrate

The change in solution from $NH_4NO_3$ to $Ca(NO_3)_2$ and then back to $NH_4NO_3$ had an insignificant effect on $NO_3^-$ fluxes ($P = 0.22$). The fluxes of $NH_4^+$ were also unchanged by the temporary removal and then replacement of $NH_4^+$. Changing the solution composition did, however, have a significant effect on the $H^+$ fluxes ($P = 0.03$). The fluxes of $H^+$ appeared to be reduced upon removal of $NH_4^+$ from the solution but this was not significant. Subsequent return of $NH_4^+$ to the solution led to an approximate doubling of $H^+$ efflux compared to when the solution did not contain $NH_4^+$ ($P = 0.03$).

***Figure 5.11.*** *$NH_4^+$ effects on $NO_3^-$, and $H^+$ fluxes. Fluxes are the means of 4 plants (± SEM, n=4). Roots were equilibrated in each solution ($NH_4NO_3$ or $Ca(NO_3)_2$) for 1 hour prior to measurement. Measurement solutions were 100 µM $NH_4NO_3$ plus 200 µM $CaSO_4$ or 50 µM $Ca(NO_3)_2$ plus 200 µM $CaSO_4$.*

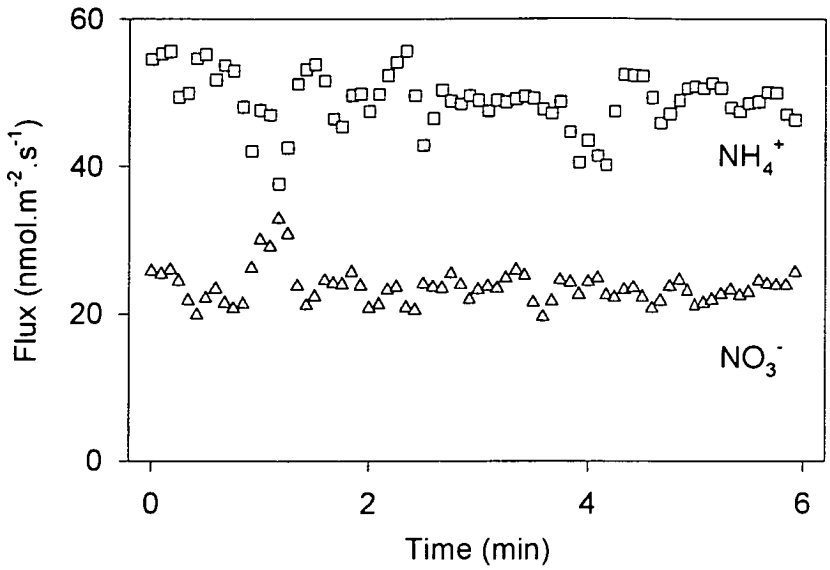



### 5.3.4 Kinetics

The timescale of measurement for the kinetics measurements was chosen to try and minimise the contribution of efflux to net flux and in this way measure influx. For this reason flux measurements were taken as soon as possible after solution concentrations had stabilised after solution changes. The time taken to change the solution concentration was approximately 4 minutes and fluxes were measured for up

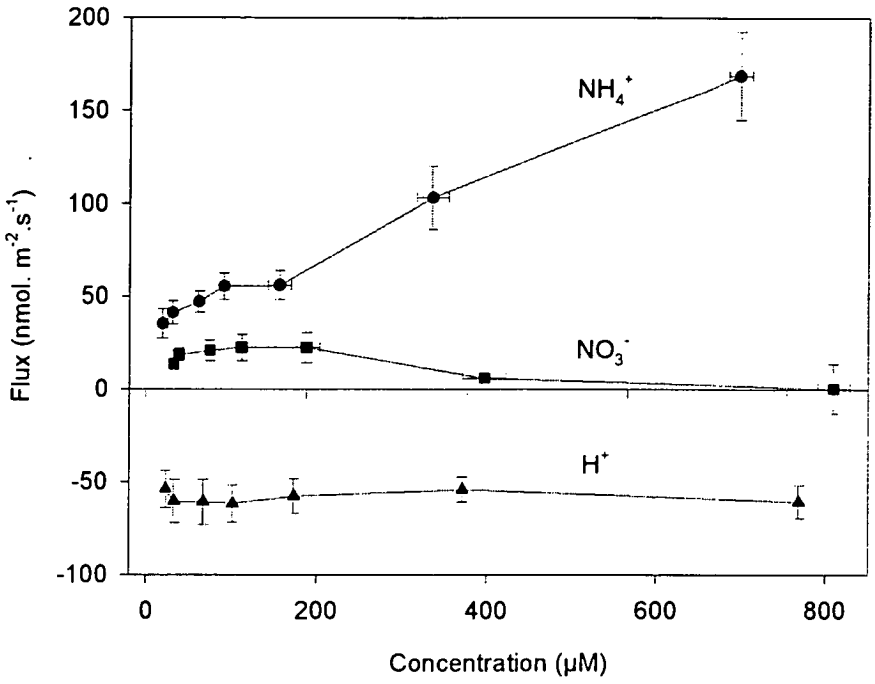
to 6 minutes after this. The fluxes measured were relatively stable throughout this period (Figure 5.12). The flux value for each concentration was taken as the average of the fluxes measured throughout this period.

***Figure 5.12.*** *Fluxes of $NH_4^+$ and $NO_3^-$ measured immediately after the solution was changed from 25 μM to 50 μM. The solution was changed by flowing 50 μM $NH_4NO_3$ through the chamber for 4 minutes.*




The concentration dependence of $NH_4^+$ and $NO_3^-$ uptake, based on the estimated concentrations at the root surface and the corresponding flux values, are shown in Figure 5.13. For $NH_4^+$ uptake there is evidence (heavily dependent on the flux measurement at 150μM) for a single saturable uptake system up to a concentration of ≈ 300 μM. Above 300 μM there was a large increase in the rate of $NH_4^+$ uptake. At 350 μM the uptake rate was 60% greater than below 300μM and at 800 μM the uptake rate was 180% greater than that at 180 μM. The $NO_3^-$ concentration dependence of uptake was not as clear as that for $NH_4^+$; there was apparent saturation below 300 μM $NO_3^-$, but above this concentration the rate of uptake decreased to almost zero.

*Figure 5.13. Fluxes of $NH_4^+$, $NO_3^-$, and $H^+$ as a function of concentration at the root surface of E. nitens seedlings of $NH_4^+$ and $NO_3^-$. Values are the pooled fluxes for 4 plants (± SEM, n=4). The concentrations used for the $H^+$ fluxes are the average of the $NH_4^+$ and $NO_3^-$ concentrations.*

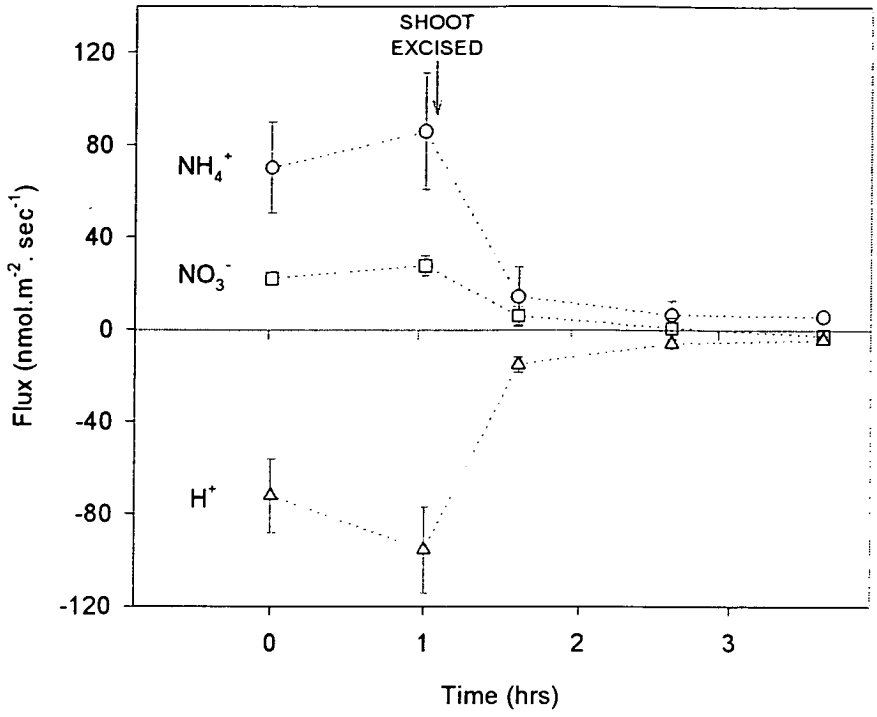



Kinetic parameters were estimated on the saturated parts of the curves in Figure 5.13, i.e. < 200 µM. The $K_m$ values were 15 µM for $NH_4^+$ uptake and 22 µM for $NO_3^-$ uptake. The $V_{max}$ values were 61 nmol $m^{-2}$ $s^{-1}$ for $NH_4^+$ uptake and 26 nmol $m^{-2}$ $s^{-1}$ for $NO_3^-$ uptake. Proton fluxes were measured simultaneously with $NH_4^+$ and $NO_3^-$ uptake and were almost constant, ranging from -53 to -60 nmol $m^{-2}$ $s^{-1}$ over the different $NH_4NO_3$ concentrations.

### 5.3.5 Excision

As shown in Figure 5.14, there was a drastic effect of excision on root fluxes. When the first post excision measurement was made, 45 minutes after excision, fluxes of $NH_4^+$, $NO_3^-$, and $H^+$ had almost completely stopped. During the two further post excision measurements there was no recovery of fluxes.

***Figure 5.14.*** *Ion fluxes before and after excision of the shoot. The values are an average of 4 plants (± SEM, n=4). For each plant the values are an average of fluxes at three positions on the root surface between 20 and 40 mm from the root tip.*




## *5.4 Discussion*

### 5.4.1 Spatial and temporal variation in fluxes

The fluxes of $NH_4^+$, $NO_3^-$, and $H^+$ within a root were relatively uniform, both spatially and temporally, in the region measured (20-50 mm behind tip). Variation within a root was smaller than that due to differences between roots (Figure 5.7). Similar to these results for eucalypts, studies with maize have found that away from the root tip, uptake of ions is uniform along the primary root axis (Marschner et al., 1991; Lazof et al., 1992). Zobel et al. (1992) also using a MIFE system, found spatial variability in $NO_3^-$ fluxes in tomato roots that showed trends in activity along the axis (5 - 40 mm) of different types of root (tap, basal, and lateral) that changed with age, and total root surface area. It may be that the eucalypt roots used in the present study were not old enough to exhibit variation of this nature, or that the region of measurement was too small. Other MIFE measurements on maize and barely roots have found spatial variability but the patterns were not consistent with time (Ryan et al., 1990; Henriksen et al., 1990, 1992).

Neither proximity to root hairs, or root hair density greatly affected flux rates despite the belief that root hairs are a major absorbing surface for roots in soil (Marschner, 1995). These results suggest that fluxes due to root hairs are small compared with the primary root surface. There have been root hair fluxes measured that have been relatively low. Jones et al. (1995), also using a MIFE system, found $H^+$ efflux around the 600 μm long root hairs of *Limnobium* which were at maximum 30 nmol $m^{-2}$ $s^{-1}$. If the $H^+$ flux around *E. nitens* root hairs were of similar magnitude to this then the contribution of root hairs to $H^+$ efflux would be disguised by the magnitude of $H^+$ efflux (150 nmol $m^{-2}$ $s^{-1}$) from the surface of the tap root. Zobel et al. (1992) using a MIFE system also found $NO_3^-$ fluxes at the surface of root hairs of tomato that were almost an order of magnitude less than fluxes on actual roots.

The reason for low $H^+$ fluxes found by Jones et al. (1995) and proposed here could lie in the nature of the roots measured. The roots in both cases were grown in well mixed solution culture, in which there would be little benefit, in terms of increased root radius, of root hairs because the whole root surface had access to nutrients in solution (Clarkson, 1991). As plants have been shown to vary uptake activity of regions of root in response to nutrient availability (Drew and Saker, 1984; Jackson et al., 1990; Robinson, 1994) it is possible that root hair activity is reduced when plant requirements can be met by the root surface proper. This theory is consistent with results from ethylene modified barley roots in solution culture where increased root hair density did not affect ion uptake in solution cultured plants (Crossett and Campbell, 1975).

Although the fluxes around root hairs may have been smaller than expected, the presence of root hairs adds to the error of estimated fluxes. The equations used to calculate fluxes assume the absorbing surface is an infinitely long cylinder, free of root hairs, which was clearly not the case here. Depending on the density of root hairs and their activity, the actual root surface area would have been much greater and flux estimates may have been underestimated.

Proximity of lateral roots did not appear to affect fluxes either, but it is also not clear whether they would be expected to influence flux rates of the primary root. Emerging lateral roots, like root tips generally, have high fluxes of $H^+$ and $Ca^{++}$ (Miller et al., 1988), which are associated with elongation. There is little to suggest an

involvement of $NH_4^+$ and $NO_3^-$ fluxes in elongation activity and only small fluxes of $NO_3^-$ have been measured near root tips of both maize and tomato (Lazof et al., 1992; Zobel et al., 1992). However, increased fluxes of both $NH_4^+$ and $NO_3^-$ were found near the apex of spruce roots (Marschner et al., 1991). Apart from elongating tips, lateral roots would only exert an influence on measured fluxes if the fluxes around them were much different to those of the main root surface, which does not appear to be the case. Differences in abundance of lateral roots in spruce had relatively small effects on $NH_4^+$ and $NO_3^-$ uptake by spruce roots (Marschner et al., 1991). This is supported by studies of maize roots where high rates of $NO_3^-$ uptake by lateral roots were due to the large total surface area of lateral roots and not increased uptake per unit of root surface (Lazof et al., 1992). The MIFE system could be adapted to oscillate parallel to the main root surface and in this way characterise the fluxes surrounding both root hairs and lateral roots.

There was little variation in fluxes between the two measurement times (45 - 90 min apart) at each position (Figures 5.7 and 5.8). Differences between flux measurements at the same position may in-part be attributable to slight differences (<5 μm) in repositioning the electrodes with respect to the root. In contrast, measurements of Ryan et al. (1990) using the MIFE system found a high degree of both spatial and temporal variability in fluxes of $K^+$, $Ca^{++}$, and $H^+$ along maize roots. They describe fluxes with considerable replicates where the relative occurrence of influx or efflux was about 50%. Although the relative extent of variation was less than that found by Ryan et al. (1990), Henriksen et al. (1992) found high temporal variability in fluxes in barley roots. The reason for the variability found by both Ryan et al. (1990) and Henriksen et al. (1990, 1992), but not found here with eucalypt roots could lie in the nature of the plant material used. Ryan et al. (1990) measured fluxes along three day old maize roots grown in $CaSO_4$. The eucalypt roots measured were ≈ 4 weeks old and grown in full nutrient solution. Watt and Cresswell (1987) found that in maize very little exogenous N was utilised within 7 days of germination. Hence, it is likely that the 3 day old maize plants used by Ryan et al. (1990) had low requirements for other exogenous nutrients as well. Apart from in the elongating region, fluxes would be expected to be low and simply reflecting maintenance functions. Eucalypt seeds are about 250 times smaller than maize seeds and, based on preliminary growth experiments, are dependent on exogenous N sources after ≈ 12

days. At the time of measurement (27 days) the roots would be expected to be fully functional with respect to nitrogen uptake and metabolism. This difference between the two plant tissues is also evident in the magnitude of fluxes, with the maize $H^+$ fluxes being approximately three times smaller than those found for eucalypts. Henriksen et al. (1990, 1992) measured $NH_4^+$ and $NO_3^-$ fluxes in 7-day old barley roots. Barley has a smaller seed than maize but it is still much larger than a eucalypt seed. So it is possible that the 7 day old barley roots also may not be fully functional with respect to nutrient uptake. They may be capable of taking up nitrogen but they may not be exporting nitrogen to the shoot at a high rate and this might have lead to the variable fluxes found by Henriksen et al. (1990, 1992).

#### *5.4.1.1 Stoichiometry*

There were strong correlations between the fluxes of $NH_4^+$, $NO_3^-$ and $H^+$ (Figure 5.9, Table 5.1). The strongest correlations were between fluxes of $NH_4^+$ and $NO_3^-$, and $NH_4^+$ and $H^+$. The weak correlation between $NO_3^-$ and $H^+$ fluxes could be due to the relationship being overshadowed by the higher magnitude of the $NH_4^+$ fluxes. Other simultaneous measurements of different ion fluxes with MIFE systems have not found correlations between ions. For example, Ryan et al. (1990) found no correlation between $K^+$, $Ca^{++}$, and $H^+$ fluxes in maize roots. Likewise, Newman et al. (1987), again with maize roots, found no correlation between $K^+$ and $Ca^{++}$ fluxes.

Based on present knowledge of $NH_4^+$ and $NO_3^-$ uptake and assimilation (see Chapter 2) it was expected that there should be some correlation between N fluxes and $H^+$ fluxes. The correlation between $NH_4^+$ and $H^+$ fluxes is circumstantial evidence for the localised coupling of $NH_4^+$ and $H^+$ fluxes. Assuming $NH_4^+$ uptake at low concentration is via a $H^+$ symport, either one or two $H^+$ move into the root for every $NH_4^+$ ion taken up. This would result in a net positive charge moving inwards of either 2 or 3. The energy source for this uptake is the $H^+$ motive force across the plasma membrane. To maintain the $H^+$ motive force, the $H^+$-ATPase pumps out one or two $H^+$ to make up for the one or two $H^+$ moving in with the $NH_4^+$ ion. After the $H^+$ fluxes are balanced, there is still a single net positive charge inwards which must be balanced by the excretion of some counter cation (e.g. $H^+$, $Ca^{++}$, or $K^+$) or influx of an anion (e.g. $NO_3^-$ or $Cl^-$). If $NH_4^+$ influx is electrogenically downhill via a uniport then 1 positive charge moving outwards will be needed to balance the influx.

Assimilation of $NH_4^+$ results in the production of one $H^+$ inside the root resulting in two net positive charges inside the cell for the uptake and assimilation of $NH_4^+$.

The $NO_3^-$ HATS is thought to be via a $2H^+$ symport (Glass et al., 1992, Meharg and Blatt, 1995) which results in one net negative charge inwards that must be balanced by either the influx of a positive charge (e.g. $NH_4^+$, $H^+$, $Ca^{++}$, or $K^+$) or the efflux of a negative charge (e.g. $Cl^-$). Assimilation of $NO_3^-$ leads to the production of one $OH^-$ (Raven and Smith, 1976) resulting in a net charge of two negative inwards for the uptake and assimilation of one $NO_3^-$ ion.

If the uptake rates of $NH_4^+$ and $NO_3^-$ were equal then the combined processes would be electrically neutral. The uptake ratio found for $NH_4^+$, $NO_3^-$ and $H^+$ was 3.1 : 1: -6.0 so this would lead to 4.2 (2.1 $NH_4^+$ after 1 $NO_3^-$ is subtracted, multiplied by 2) positive charges needed to be removed from the roots once $NO_3^-$ uptake was taken into account. The proportion of $H^+$ efflux found was 6.0 so there was an apparent surplus of $H^+$ moving outwards. Although it is assumed that effluxed $H^+$ is the balancing ion for $NH_4^+$ uptake, it seems unlikely in the long term (several days) as it would lead to an unsustainable increase in cytoplasmic pH. The cytoplasm has a large $H^+$ buffering capacity (Felle, 1988) so in a relatively short-term experiment (several hours) such as these, a high $H^+$ efflux may be possible without changing cytoplasmic pH. Hence, I speculate that if the measurement solution had been the same as the growth solution (with counter ions like $K^+$ available) the $H^+$ efflux may have been less.

A complication for this reasoning is that the $H^+$ efflux measured cannot be regarded as just being due to $NH_4^+$ and $NO_3^-$ uptake processes (Newman et al., 1987). Respiration within the root to maintain root processes (i.e. nutrient uptake) produces $CO_2$. The magnitude of respiratory $CO_2$ production can be greater than nitrogen uptake (Bloom and Caldwell, 1988). Excess $CO_2$ in the cytoplasm exists as carbonic acid ($H_2CO_3$), which dissociates forming $H^+$, the disposal of which will also create a $H^+$ efflux. Perhaps it is this respiratory $H^+$ efflux that accounts for the $H^+$ efflux surplus to that explained by nitrogen assimilation. Efflux of organic acids as exudates from the roots would also cause errors in estimates of proton efflux (Newman et al., 1987). Hence, in a system such as that used here, it would be necessary to quantify $K^-$

and $Ca^{++}$ fluxes simultaneously with $NH_4^+$, $NO_3^-$, and $H^+$ fluxes to gain further insight into the mechanisms of N uptake and charge balance.

### 5.4.2 pH

Changing pH from pH 4 to pH 6 affected $NO_3^-$ and $H^+$ fluxes but did not affect the $NH_4^+$ flux (Figure 5.10). Effects of pH on $NH_4^+$ uptake by other species have been various, with reports of reduced pH inhibiting or stimulating $NH_4^+$ fluxes (Marcus-Wyner, 1983; Wang et al., 1993b; see Chapter 4). This result with eucalypt roots agrees with that found by Wang et al. (1993b) with rice, where changing pH between 4.5 and 6 had no effect on $NH_4^+$ influx rates. As with $NH_4^+$ there are mixed reports as to the effects of pH on $NO_3^-$ uptake (Doddema and Telkamp, 1979, Vessey et al., 1990, see Chapter 4). In measurements using a MIFE system and maize roots, McClure et al. (1990) found that $NO_3^-$ uptake rates were highest at low pH, a result not mirrored here with *E. nitens*.

Reducing the solution pH led to a large increase in the $H^+$ efflux (Figure 5.10). This stimulation of efflux is the opposite of what would be expected from considering the driving forces for $H^+$ flux. Changing the external pH from pH 6 to pH 4 corresponds to changing the $H^+$ concentration from 1 µM to 100 µM. Based on the internal pH remaining stable with this external pH change (Felle, 1987, 1988) the driving force for $H^+$ influx is much greater at pH 4 than at pH 6.

The high $H^+$ efflux might be explained by considering the timescale of the measurements. That is, one could speculate that the high efflux at low pH, in this case measured after an hour, was a response to a high initial influx once the pH was reduced from pH 6 to pH 4. The high efflux could have been a reflection of cellular processes stabilising cytosolic pH. However, this explanation is not consistent with the results of some incidental measurements. On three occasions when the solution was changed from pH 4 to pH 6 a flux measurement was made as soon as the new pH had stabilised, i.e. between 5 and 10 minutes after the previous measurement and at the same position. In each case the $H^+$ flux showed the same trend as the measurements an hour later, i.e. a decrease in $H^-$ efflux with an increase in pH. In contrast to $H^+$, $NH_4^+$ and $NO_3^-$ fluxes measured on a short time scale were different to those measured later. In each case the $NH_4^+$ flux was reduced in the short term but

recovered to the initial value by the time of the measurement an hour later. The $NO_3^-$ flux increased upon the pH changing to 6 and stayed at this level until the hour measurement.

A combination of short and long term flux measurements after changing solution pH would be required to understand the dynamics of the root response to changing pH.

### 5.4.3 Ammonium inhibition of nitrate uptake

The removal of $NH_4^+$ from solution did not lead to an increase in $NO_3^-$ flux (Figure 4.11) as would be expected if $NH_4^+$ inhibited $NO_3^-$ uptake as has been found in a number of other plant species (rye grass and clover, Macduff and Jackson, 1992; barley, Rao and Rains, 1976; Ayling 1993; Douglas fir, Kamminga-Van Wijk and Prins, 1993; and others, see Chapter 4). This result with eucalypts agrees with that of McClure et al. (1990), who in maize found no effect of $NH_4^+$ on $NO_3^-$ flux in experiments where $NH_4^+$ exposure was for less than 1 hour. It is possible that in the experiments here, the removal of $NH_4^+$ from solution needed to be for a longer period to have affected $NO_3^-$ fluxes. Lee and Drew, (1989) found that growing barley on $NH_4NO_3$ made $NO_3^-$ influx insensitive to the presence of $NH_4^+$. Hence, it could be that growth of eucalypts on $NH_4NO_3$ removed sensitivity of $NO_3^-$ uptake to $NH_4^+$.

### 5.4.4 Kinetics

Ammonium uptake (Figure 5.13) followed the classical relationship with concentration (Epstein, 1972). At low concentration (<200 μM) there was a saturating system corresponding to a high affinity transport system (HATS, either via an $NH_4^+$ uniport or a $H^+$ symport) with a $K_m$ of 15 μM and an $V_{max}$ of 62 nmol $m^{-2}$ $sec^{-1}$. At high concentration (>300 μM) there were only two data points but the increase in uptake at these concentrations was consistent with a linear increase characteristic of a low affinity transport systems (LATS, perhaps via $K^+$ channel or a specific $NH_4^+$ channel). The results follow quite closely the concentration dependence found for $NH_4^+$ influx for another tree, spruce, by Kronzucker et al. (1996).

Nitrate uptake also follows classical saturation kinetics at low concentration corresponding to a HATS with a $K_m$ of 22 μM and $V_{max}$ of 26 nmol $m^{-2}$ $sec^{-1}$. As with

the results of Kronzuker et al. (1995d, 1996), saturated uptake rates were much less than those found for $NH_4^+$. The eucalypt plants used here were grown in the presence of $NO_3^-$, so the $NO_3^-$ uptake here should correspond to the induced state of the HATS. For $NO_3^-$-induced seedlings, Kronzucker et al. (1995d, 1996) found $V_{max}$ values for $NO_3^-$ were a quarter of those for $NH_4^+$ whereas in this study the $V_{max}$ for $NO_3^-$ was just over half that for $NH_4^+$. A higher proportion of $NH_4^+$ uptake than found here was also found in *Lolium sp.* when grown with both $NH_4^+$ and $NO_3^-$ (Clarkson and Warner 1979, Clarkson et al., 1986).

At higher concentrations, $NO_3^-$ uptake did not follow the pattern shown here for $NH_4^+$, and found by others for $NO_3^-$ (Siddiqi et al., 1990; Aslam et al., 1992; Kronzucker, et al., 1995d). In the concentration range usually corresponding to the LATS, uptake rates were reduced to almost zero. This reduction in uptake would appear to correspond to an inhibition of $NO_3^-$ uptake by $NH_4^+$. One of the reasons $NH_4NO_3$ was used in this experiment was that, as shown in the previous section, the removal of $NH_4^+$ from solution did not change $NO_3^-$ fluxes. The results shown in Figure 4.13 appear contrary to this non-inhibition and suggest that $NH_4^+$ is inhibiting $NO_3^-$ uptake but only at concentrations greater than 100 μM. An inhibition only at higher concentrations (>100 μM) does not agree with the findings of Lee and Drew (1989) who found that $NH_4^+$ inhibited $NO_3^-$ uptake at concentrations as low as 5 μM.

An alternative explanation for reduced $NO_3^-$ uptake at high concentration may be in terms of uptake regulation. Concentrations were increased stepwise throughout the range of concentrations and flux measurements were made soon after concentration changes to limit equilibration between the cytoplasmic pool and the external solution. A problem with this approach is that the cytoplasmic pool at higher external concentrations, although not at equilibration, is higher than at lower concentrations. If the cytoplasmic concentration (Ci) has a role in regulating $NO_3^-$ uptake (Siddiqi et al., 1989; King et al., 1993) then the reduction in $NO_3^-$ uptake could be reflecting this regulation. Alternatively, the increase in the cytoplasmic concentration during the time of the early concentration changes could have saturated other $NO_3^-$ transport and assimilatory processes and lead to a later reduction in $NO_3^-$ uptake processes. These explanations may not be valid as high concentration uptake via the LATS is thought to not be subject to regulation (King et al., 1993).

The flux of $H^+$ was practically constant for the entire concentration range of $NH_4NO_3$ (Figure 5.13). There was no correlation between $H^+$ flux and the increase in $NH_4^+$ flux which occurred at higher concentration. This asynchrony could be reflecting the different mechanisms responsible for increased uptake. For example, at high concentrations (>200μM) $NH_4^+$ uptake is thought to be passive, perhaps via $K^+$ channels (Ullrich, 1992). This uptake would not be directly linked to $H^+$ fluxes as they would in a $NH_4^+/H^+$ symport.

According to the findings of Ritchie and Pravan (1996) there were not enough points in the saturating concentration range (< 200 μM) to accurately estimate kinetic parameters (Ritchie and Pravan suggest at least 10 are required). This factor could and would be incorporated into further experiments but as they now stand the current estimates appear as robust as the majority in the literature. One aspect of the saturation curves that cannot be improved are points at very low concentrations. Low concentration measurements are limited by the detection limits of the electrodes ($NH_4^+$ 1 μM; $NO_3^-$ 30 μM, Henriksen et al., 1990). The detection limit for $NO_3^-$ precludes measurements of $C_{min}$ and the lower range of estimates of $K_m$ (approximately 15 μM, see Table 3.1). However, for both ions it is possible to measure uptake in the region of $K_m$ and up to the concentration where uptake is saturated (approximately $V_{max}$). If the concentrations where uptake is measured were evenly spaced within this region then it should be possible to provide reliable estimates of both $K_m$ and $V_{max}$ (Ritchie and Pravan, 1996). Hence, despite limitations at low concentrations, the MIFE technique should continue to be a satisfactory technique for describing the majority of the concentration dependence of $NH_4^+$ and $NO_3^-$ uptake.

These measurements of the concentration dependence of uptake were based on the assumption that the fluxes measured in the 4-10 minutes after concentration changes commenced would be almost equivalent to influx. As shown in Figure 5.12, fluxes were relatively stable throughout the measurement period. It was assumed that if measurements had continued for a longer time, fluxes would have reduced as the cytoplasm equilibrated with the external solution at each new concentration. A proper study of the dynamics of these possible changes would lead to a better understanding of the kinetics results.

### 5.4.5 Excision

These results show that excision has rapid dramatic effects on the fluxes of $NH_4^+$, $NO_3^-$, and $H^+$ (Figure 5.14). Bloom and Caldwell (1988) reported results where the effect of excision was quantified for a greater number of ion fluxes plus some gas fluxes. Their results were similar in overall effect to those presented here although the maximum depression of fluxes took approximately 4 hours as opposed to almost 1 hour as shown in Figure 5.14. This reduction in flux is at odds with the findings of Huang et al. (1992) who found that excision had little effect on potassium influx in the short term.

The main difference between the experiments of Huang et al. (1992), and those of Bloom and Caldwell (1988) and the results presented here is that the former measured unidirectional influx into low salt roots. Whereas, in the latter, net uptake was measured in roots which were in what approximated to a steady state for the nutrient in question. Huang et al. (1992) pre-treated their intact roots overnight in $CaSO_4$ so that the cytoplasmic pool of $K^+$ was greatly reduced. Their uptake measurements probably reflected simple equilibration between the cytoplasm and the external solution in a short enough time frame to exclude significant efflux, i.e. a unidirectional influx. In the nitrogen results presented here, and in those of Bloom and Caldwell (1988), the plants were pre-treated overnight in the same concentration as that in which they were measured. With such pre-treatment, the cytoplasm would have been in equilibrium with the external solution and internal pools would have been saturated. It could be assumed that apart from some N transport to the vacuole, which would already be at or near equilibrium, and some N utilised for root growth, most N taken up would be transported to the shoot. Once the root is excised, the sink, i.e. the shoot, is removed and without a sink to which N could be transferred, the uptake processes could have been reduced. Aslam et al. (1996), also found that there was little effect of excision on ion influx and that excision stimulated efflux and reduced net uptake. This result agrees with an hypothesis that the steady state response is due to removal of the sink for nutrients rather than a cessation of potential activity by uptake mechanisms.

Gas fluxes measured by Bloom and Caldwell (1988) also agree with the proposition that excision leads to a general slowing down of root processes. After

excision, Bloom and Caldwell found that fluxes of $O_2$ and $CO_2$ were reduced to approximately half their pre-excision values, but did not completely cease. As $H^+$ fluxes were reduced on the same scale as $NH_4^+$ and $NO_3^-$ uptake, this hypothesis points to the steady state $H^+$ fluxes being due to $NH_4^+$ and $NO_3^-$ uptake, and not simply to an unrelated influx.

It is likely that, if efflux had been minimised by depleting the cytoplasmic $NH_4^+$ and $NO_3^-$ pools, the measured net fluxes may not have shown the rapid decrease after excision as shown in Figure 5.14. Unless this theory was validated, the use of excised roots in nutrient uptake experiments is not justified.

## *5.5 Conclusions*

Within the region 20-50 mm from the root tip of *E. nitens* seedlings, spatial and temporal variability of $NH_4^+$, $NO_3^-$, and $H^+$ fluxes was relatively low. The ratio of these fluxes in this region was constant, according to the ratio 3.11:1:-5.96 ($NH_4^+$:$NO_3^-$:$H^+$). The presence or absence of $NH_4^+$ had no affect on $NO_3^-$ fluxes. Changing solution pH from pH 6 to pH 4, did not affect $NH_4^+$ fluxes, reduced $NO_3^-$ fluxes, and greatly increased $H^+$ efflux.

The concentration dependence of $NH_4^+$ fluxes showed evidence of a saturating HATS below 200 μM with a $K_m$ of approximately 15 μM. Above 300 μM there was some evidence for a LATS system with fluxes increasing linearly with increasing concentration. At concentrations below 200 μM, $NO_3^-$ fluxes were about half those of $NH_4^+$ and also showed evidence of a saturating HATS with a $K_m$ of approximately 20 μM. Above 300 μM, $NO_3^-$ fluxes were reduced, possibly due to inhibition by $NH_4^+$ or negative feedback of high cytoplasmic $NO_3^-$ concentrations.

Shoot excision led to a rapid, dramatic, reduction in $NH_4^+$, $NO_3^-$, and $H^+$ fluxes, which suggests that excised roots should only be used with extreme caution to measure nutrient uptake processes in roots.

# 6. Discussion

## *6.1 Comparison of N uptake measured using the MIFE and depletion methods in the present study*

### 6.1.1 Magnitude of fluxes measured

The depletion and MIFE experiments represented two different ways of measuring $NH_4^+$ and $NO_3^-$ uptake by *E. nitens* roots. The main difference between the two sets of experiments was that, in the depletion experiments, $NH_4^+$ and $NO_3^-$ uptake of the whole root system was measured, whereas, in the MIFE experiments, uptake was measured over one small area of the tap root. For this reason the results are not directly comparable. However, it was possible to estimate total uptake by roots of the MIFE plants by making some assumptions about uptake rates across the whole root system.

The simplest method to estimate uptake rates for the whole root system was to multiply uptake rates found for the 20-50 mm region of the primary root by the total root size. Firstly, the MIFE uptake rate (nmol $m^{-2}$ $s^{-1}$) needed to be converted to a fresh weight basis (depletion uptake rates were generally in units of μmol $gFW^{-1}$ $hr^{-1}$). The surface area to weight ratio of the roots was 154 $cm^{-2}$ $gFW^{-1}$. Using this ratio, the $NH_4^+$ uptake rate estimated for the MIFE plants was approximately 3.9 μmol $gFW^{-1}$ $hr^{-1}$ for uptake measured at 100μM. This value is within the range, but at the top end, of the measurements found using the depletion method (range 1.5 to 4.5 μmol $gFW^{-1}$ $hr^{-1}$). This value, however, is probably an overestimate because nitrate absorption rates in lateral roots are roughly a third of those found on the primary root of some species (Lazof et al., 1992). The validity of this estimate could be improved by estimating the proportions of different root types and measuring the uptake rates of lateral roots with the MIFE technique.

The concentration dependence of $NH_4^+$ uptake found with the MIFE technique was similar to that found for $NH_4^+$ influx by spruce using $^{13}N$ (Kronzucker et al., 1996). In both cases there was a saturable system below 300 μM and a linearly increasing system above 300 μM. The concentration dependence curves based on the depletion technique did not follow this pattern, but instead were similar to that found

by others using the depletion technique for $NO_3^-$ (Laine, et al., 1993; Swiader and Freiji, 1996).

A major difference between the two different types of curves is the absence of evidence for a low affinity transport system for $NH_4^+$ using the depletion method. To my knowledge, there has only been one occurrence in the literature of a LATS being evident from depletion experiments and this was for nitrate in maize (Aslam et al., 1992). The measurement of LATS uptake using the depletion method is discussed later in this Chapter (6.2).

### 6.1.2 pH effects

The pH effects on $NH_4^+$ an $NO_3^-$ uptake differed for the two approaches. Using the depletion method, uptake of both $NH_4^+$ and $NO_3^-$ was higher at pH 4 than pH 6 (Figure 4.4). Whereas, with the MIFE technique, uptake of $NH_4^+$ was unaffected by pH, and $NO_3^-$ uptake was halved at pH 4 compared with pH 6 (Figure 5.10).

The reasons for the difference between the two methods are unknown but may be due to differences in the experimental protocols. There was a difference between the culture methods of the plants used in each method in that the plants were grown at different solution pH. Plants used in the depletion experiments were grown for more than twice as long as the MIFE plants. In this time the pH of the culture solutions was reduced rapidly by the growing plants. Daily pH adjustment was required to keep the pH at just above pH 4. The MIFE plants were much smaller than the depletion method plants and did not have the dramatic effects on solution pH that the larger depletion plants had. The unadjusted pH of the nutrient solution was stable at pH 5.8 and did not need adjustment between twice weekly solution changes. In hindsight the nutrient solution of the MIFE plants should have been adjusted to pH 4 at solution changes. This difference in culture pH could have caused the differences found in the different methods but this assertion would need to be experimentally verified.

Another possible cause of differences in pH response between the two methods is that the depletion experiments used high buffer concentrations to stabilise pH whilst the MIFE experiments where unbuffered. As pointed out in the discussion to Chapter 4 the different buffers used at pH 4 and pH 6 (MES pH 4, tartaric acid pH 6) could have led to effects on root physiology.

The MIFE pH result was limited in that it was based on one measurement made 1 hour after a change in the solution pH. In view of the unusual and seemingly unsustainable effect the pH change had on the $H^+$ fluxes, the MIFE result is not conclusive and the temporal nature of this effect would need to be investigated. Hence, the pH results found in the depletion experiments would appear to be more reliable.

### 6.1.3 Ammonium inhibition of nitrate uptake

There was an apparent inhibition of $NO_3^-$ uptake by the presence of $NH_4^+$ in the MIFE experiments, but only when the concentration of $NH_4NO_3$ was above 200 μM. At 100 μM $NO_3^-$, the presence or absence of $NH_4^+$ did not effect $NO_3^-$ uptake. This result implies that the inhibition of nitrate uptake by the presence of $NH_4^+$ occurs only at concentrations above the level of saturation of the HATS.

In the depletion experiments there was no inhibition of $NO_3^-$ uptake by the presence of $NH_4^+$ when depletion was measured from a starting concentration of 1 mM. This is in the range of concentration in which there was inhibition in the MIFE experiments. This difference between the two methods may relate to the inability of the depletion method to measure LATS uptake in the range 300-1000 μM (Chapter 6.2). If the depletion experiments do not measure LATS uptake, and the inhibition only affects LATS uptake, then this could explain the non inhibition found in the depletion experiments.

However, although the measurement protocol used in the MIFE experiments may have enabled a relatively accurate measurement of the concentration dependence of the uptake mechanisms, this may not reflect the processes that may occur in a more long term situation. The kinetics experiment may be viewed as measuring a transient response to a change in concentration. The lack of response of $NO_3^-$ uptake to $NH_4^+$ seen in both the depletion experiments, and those of the MIFE $NH_4^+$ inhibition experiment, may be more akin to the long term affect of $NH_4^+$ on $NO_3^-$ uptake. With this in mind it would seem reasonable to assume that $NH_4^+$ does not inhibit 'steady state' $NO_3^-$ uptake by *E. nitens*.

### 6.1.4 The MIFE technique for measuring nutrient fluxes

The MIFE approach has shown itself to be a reliable method for measuring fluxes of $NH_4^+$, $NO_3^-$, and $H^+$ in plant roots. Measurement of net uptake under conditions approximating a steady state situation can be readily made. The time resolution of the MIFE approach means that root responses to treatment effects such as changing concentration and pH can be investigated with much greater accuracy than any other method that does not use radioactive tracers. The capability of the system to measure $NH_4^+$ and $NO_3^-$ uptake simultaneously further enhances the potential of the approach. The high temporal and spatial accuracy, combined with simultaneous measurement of N and $H^+$ fluxes, provides a method for investigating the mechanisms of $NH_4^+$ and $NO_3^-$ uptake in greater detail than was previously possible. However, as highlighted above the temporal resolution of the MIFE system can perhaps lead to problems of interpretation in a similar way to that of the depletion experiments.

A problem with the MIFE experiments was caused by the number of replicates used in the experiments. The variability found in experiments such as the spatial and temporal scans (5.3.1) meant that the results were somewhat compromised by the low sample number. A larger sample size would increase the applicability of this technique.

## *6.2 Reflections on the theory and practise of measuring uptake kinetics by various methods*

### 6.2.1 Enzyme kinetics vs. uptake kinetics

The reason why nutrient uptake tends to follow enzyme kinetics is because nutrient transporters are enzymatic in their action. Instead of catalysing a reaction between substrates they catalyse the transport of a nutrient across a membrane (Epstein and Hagan, 1952). What complicates the matter is that enzyme kinetics describe enzyme action when an enzyme has free access to the substrate. In plant roots the enzymes (transport proteins) are in the root plasma membrane and are just one part of the nutrient uptake process. The issue is confused by the complex root structure, intracellular pools that saturate and long term assimilatory processes that

remove nutrients. So, although the uptake transporters may be enzymatic, because we are dealing with roots and not a purified enzyme extract, the characterisation of their activity is complicated.

### 6.2.2 Uptake kinetics measured via the different measurement techniques

#### *6.2.2.1 Unidirectional influx experiments*

Unidirectional influx experiments attempt to measure the transport of nutrients, across the plasma membrane, into the cytoplasm . These experiments estimate influx as distinct from net fluxes where net fluxes can include an efflux component. These influx measurements are made with a radioactive tracer such as $^{13}N$, and utilise experimental protocols that minimise efflux. Part of these studies involve a characterisation of the compartmental characteristics of the root using half times of exchange. The half time of exchange is an estimate of the time required for the turnover of pools within the root such as the surface film, the free space (apoplasm), the cytoplasm, and the vacuole. The cytoplasmic half time of exchange is used to decide the time for which the $^{13}N$ label is supplied to allow measurable flux into the cytoplasm but will minimise efflux of the $^{13}N$ label. The free space and surface film half-lives are required to decide upon a rinsing time such that $^{13}N$ label that is not in the cytoplasm can be removed from the root so that it does not lead to overestimation of the influx. This method allows an instantaneous 'snapshot' of the influx characteristics of the root, without any changes to the nutrient background. Plants can be growing in 100μM $NH_4^+$ and the unlabelled $NH_4^+$ can be replaced by labelled $NH_4^+$ when influx measurements commence.

This method when applied to estimate uptake kinetics reflects the actual kinetics of transport across the plasma membrane. In this way they are direct estimates of the kinetic behaviour of plasma membrane transport in the way first introduced by Epstein and Hagan (1952). In using this technique, treatment effects such as nutrient status of plants can be investigated in terms of their effect on the transporters themselves as distinct from higher level regulatory processes.

### *6.2.2.2 Depletion method*

The depletion method was first developed to offer a simple and quick means of estimating uptake kinetics (Claassen and Barber, 1974). Since it's inception, the method has been used extensively for a wide range of plant species. A problem with the method, highlighted in Chapter 3, is that the original method has been changed by various authors without proper justification and with deleterious effects on the comparability of parameters estimated using, what should be, the same technique.

Another possible problem with the depletion method for estimating uptake kinetics is in interpretation. Unlike radioactive tracer experiments, depletion experiments do not measure the concentration dependence of influx across the plasma membrane into the cytoplasm. In depletion experiments the net uptake is measured over a gradually decreasing concentration range. At each concentration the influx will be determined by the concentration at the plasma membrane, but there are a number of other processes that will concurrently affect net uptake. The most important of these processes will be efflux from the cytoplasm to the external solution. In barley plants grown under similar conditions as the eucalypts of the depletion experiments in the present study, and with uptake rates measured via depletion from 200 μM, efflux rates were 70% of the measured unidirectional influx rates (Lee, 1993). The rate of efflux will be determined by the extent to which influx is surplus to plant requirements. The plant requirements are represented by the flux from the cytoplasm to the vacuole, and assimilation and transport to the shoot. In this way, net uptake will be dependent on the plant requirements more than the strictly kinetic response of transporters to external concentration.

As an example, in some of the depletion experiments presented here, net $NH_4^+$ uptake of a plant was measured by depletion of solution from 100μM to near zero. The influx rate to the cytoplasm would have been determined by the external concentration according to influx kinetics. Inside the cytoplasm, $NH_4^+$ would have been transported to the vacuole or assimilated and transported to the shoot, $NH_4^+$ surplus to these requirements would return to the external solution as efflux. In this way the rate of depletion of the solution will actually reflect utilisation by the plant and not uptake kinetics. As the external concentration dropped, utilisation rate equals and becomes greater than the influx rate, the concentration dependence of uptake will

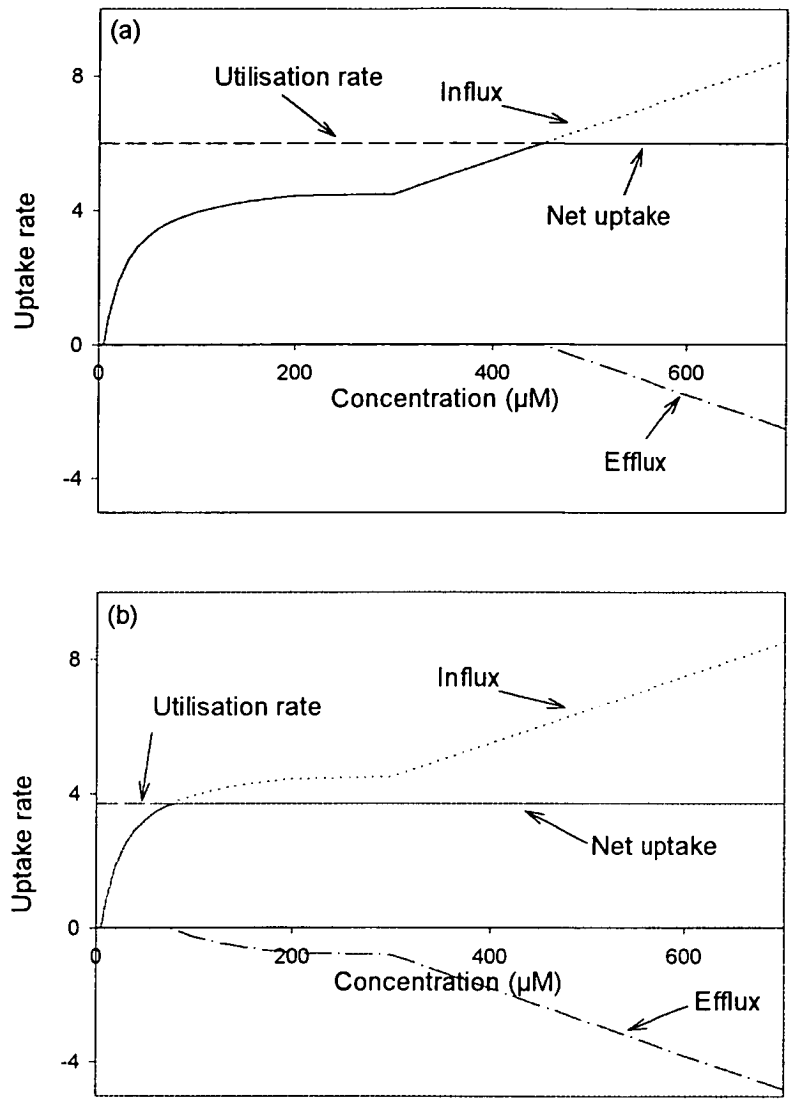
be reflected in the concentration dependence curve. This example assumes that the plants being previously grown at 100 μM $NH_4^+$, the roots will be equilibrated to this concentration and flux to the vacuole will be minimal.

This example highlights problems of the technique. If the $V_{max}$ of the transporters is higher than the maximum utilisation rate then the $V_{max}$ measured via depletion will be this utilisation rate and not the $V_{max}$ of influx. If this utilisation rate is higher than the $V_{max}$ rate then the depletion curve will describe the $V_{max}$ of uptake. A graphical representation of these processes is outlined in Figure 6.1. One point shown in these graphs is that the $K_m$ in both situations will be within a relatively small concentration range.

In Figure 6.1 (b) the utilisation rate is high enough to lead to the LATS being discernible. It is unknown how viable this scenario is. There appears to be only one case in the literature where the LATS has been evident in an N depletion experiment, so it is possible that it occurred in this case (Aslam et al., 1992).

Regardless of the problems of estimation (Chapter 3) and interpretation, perhaps the most telling problem with the depletion method is the duration of a depletion experiment. Many depletion experiments run for as long as 10 hours, presumably because, as was the case in experiments of Chapter 4, it is difficult to optimise the root to solution volume ratio (see depletion times, Table 3.1). Claassen and Barber (1974), in the initial description of the depletion technique suggest that to reduce chances of diurnal variation the time required for full depletion should be kept to within 3 to 5 hours. Evidence relating to the timescale of plant responses (Doddema and Otten, 1979; Clarkson, 1986; Jackson and Volk, 1992) suggests that not only diurnal variations could be important but also root responses to changing concentrations within the timescale of the experiment.

***Figure 6.1*** *Hypothetical fluxes involved in depletion experiments: (a) Depletion when utilisation rate is greater than the $V_{max}$ for influx, and (b), depletion when utilisation rate is less than the $V_{max}$ for influx.*




### *6.2.2.3 Net uptake at discrete concentrations*

Discrete concentration uptake measurements include all those where net uptake is measured at different distinct concentrations. These types of experiments fall into two groups, either short- or long-term. Long-term variable concentration experiments are perhaps the furthest removed from the original method for estimating root uptake kinetics described by Epstein et al. (1963). This method involves growing plants at different constant concentrations and the uptake is taken as the incremental increase in nutrient amount in the plants over the growth period (Wild et al., 1979; Mullins and Edwards, 1989). This method may yield a concentration versus uptake curve but it is

not in the same context as in other experiments. Plants growing at different concentrations would have different nutritional status, thus each plant at each concentration could have a different set of uptake kinetics (Siddiqi et al., 1990, Wang et al., 1993b). If used for N it cannot distinguish between $NH_4^+$-N and $NO_3^-$-N unless used in conjunction with a tracer such as $^{15}N$.

The other methods used to measure net uptake at discrete concentrations do so over relatively short periods. The MIFE experiments investigating kinetics in Chapter 5 fit into this category. Plants were grown and then uptake measured over a range of concentrations starting at low concentrations and moving up to higher concentrations. The time taken to measure at each concentration differs between authors. In the case of the MIFE experiments, the time to complete the full range of concentrations was minimised (< 80 min) in order to avoid equilibration at each concentration. Other investigators utilising this technique have not focused on the time taken between concentration changes and measurement, and simply get the concentration range measured in the time required by their method to measure uptake (Rao and Rains, 1976; Raman et al., 1995a, 1995b). In another technique, roots are given time to reach a steady state at each new concentration (up to 3 hours) before measuring uptake (Bloom, 1985; Smart and Bloom, 1993). As with the depletion method the timescale of these experiments may have significant effects on the results due to changing root properties.

The MIFE kinetics experiments of Chapter 5 tried to estimate influx. Depending on how close the assumed half times of exchange parameters and the actual exchange parameters for eucalypt roots were under the experimental conditions, the measured net uptake should reflect influx. If the time between concentration changes and measurement had been greater (10-15 minutes) the cytoplasmic compartment could have equilibrated with the external solution and efflux may have been a significant component of net fluxes.

### 6.2.3 The most appropriate method?

All of the methods discussed above have problems in their application and interpretation. The major problem with the above methods is that the differences due to what flux or uptake is actually being measured are rarely acknowledged. This

factor is in part responsible for the wide range of kinetic parameters found in the literature.

The depletion method has been widely used for estimating uptake kinetics. Of the methods described above the depletion method is the most problematical, firstly, because uptake is measured from high to low concentrations so efflux would be greatest, and secondly, because an extra step involved in fitting depletion curves adds to the likelihood of errors in estimates (Chapter 3). The depletion curves produced in this method require very precise concentration measurements especially in the range of possible $K_m$ values as highlighted by Drew et al., (1984). The universality of the depletion method would be increased if efflux could be quantified, the timescale of experiments were reduced, and if there was some standardisation of the method.

Methods that can estimate the kinetics of influx as opposed to net uptake, such as the $^{13}N$ method and perhaps the MIFE method, provide information regarding the mechanisms of uptake. In the same way as other methods described are difficult to interpret, these influx measurements are difficult to apply to roots growing in a long-term situation in solution culture, or even more difficult to apply to roots growing in soil. Hence, the most appropriate method of estimating kinetics may be dependent on it's application.

Apart from the differences due to the method by which uptake is measured, there is another major source of differences in the parameters found in the literature. In a review of kinetics estimation methods in the biochemical literature, Ritchie and Pravan (1996), found that the estimation methods in the literature were uniformly poor. It is also assumed that this would be the case in the literature relating to uptake kinetics of plant roots. The major problems in parameter estimation are, firstly, the number and spread of data points used, and secondly, the inappropriate use of Lineweaver-Burk plots without weighting different points. An improvement in parameter estimation techniques, specifically discarding the Lineweaver-Burk method in the way it is being used at present, would result in further improvement in the validity of parameter estimates (Ritchie and Pravan, 1996).

## *6.3 Applications of uptake measurements*

### 6.3.1 Kinetic parameters for process based modelling

Apart from methodological differences in estimates of kinetic parameters, uptake kinetics change due to environmental stresses such as nutrient availability and temperature (Siddiqi et al., 1990; Wang et al., 1993b; Cogliati and Clarkson, 1983; Cruz et al., 1993; Laine et al., 1993). These changes in kinetics are thought to be relatively short term responses to maintain N in internal pools at a relatively constant level (Clarkson, 1985). Long term changes to environmental stresses appear to lead to changes in growth rate and root:shoot ratios because the benefits of increased uptake capacity/affinity are limited and energetically costly (Chapin, 1988). An example of this is given in the temperature experiment in Chapter 4, where reduced temperature led to changes in $V_{max}$ in the short-term, and in the long-term different root:shoot ratios and growth rates. It was also apparent in the RGR/nutrient status experiment, where the most noticeable effect of low nutrient status was the increase in the size of the root system with respect to the shoot. Differences in uptake kinetics between plants may reflect adaptation to different environments (Chapin, 1988). The above factors lead to 'actual' differences in uptake kinetics, as opposed to the methodological differences discussed in Chapter 6.2.

Due to the 'actual' differences in uptake kinetics it is difficult to choose what parameters to use to describe uptake by roots in soil for process based uptake models such as the Barber-Cushman or COMP 8 models (Oates and Barber, 1987; Smethurst and Comerford, 1993). Ideally, different kinetics should be estimated for different contingencies. For example, for modelling conditions leading to low nutrient supply, kinetic parameters measured on plants grown under low concentrations/low RAR should be used. Unfortunately, as discussed in Chapter 6.2, methodological problems mean that we have difficulty in accurately estimating kinetics that are independent of measurement technique. In effect, the 'actual' differences in kinetics may be less than the methodological differences. Added to this problem are the differences, of uncertain magnitude, between parameters of solution cultured plants and those of plants growing in soil. In view of these points, it may be both easier and more reliable not to spend the effort on actually measuring kinetic parameters for different plants and circumstances, but to appropriate credible values from the literature. This

suggestion requires clarification as to what constitutes credible values within the vast N literature. As shown in Table 3.1, there is large variation in kinetic parameter estimates. It may be practical to take median, rather than mean, values from these estimates for use in process based models.

The parameter $C_{min}$ is not a classical M-M kinetic parameter but it is used in process based models is used as a kinetic parameter. There is much less variation in $C_{min}$ values presented in the literature than in $V_{max}$ and $K_m$ values (Table 3.1). This lack of variation is due to the simplicity of $C_{min}$ measurements which do not involve any curve fitting, simply solution concentration measurements after depletion is complete. Depending on the effect different values of $C_{min}$ may have on the output of process based models, it may be both prudent and practical to measure $C_{min}$ for individual plants and conditions.

### 6.3.2 The use of excised roots in uptake experiments

The MIFE experiments investigating excision effects on root fluxes found that excision had dramatic negative effects (Figure 5.13). It was proposed that with appropriate pre-treatments excised roots may be viable for uptake measurement. Dependent on the validation of this theory the MIFE system could prove a valuable new method for measuring N uptake activity of soil grown roots. At present the only way to measure N uptake kinetics of field grown plants is to use the non-radioactive tracer $^{15}N$, or limited circumstances where it is available, the radioactive tracer $^{13}N$. As mentioned previously, $^{15}N$ is not a suitable tracer for kinetics estimation because the loading time required to achieve adequate labelling is too long to preclude efflux (Clarkson, 1986). The MIFE system offers a reliable means of estimating kinetics in roots and, subject to the case of excised roots being a valid method, could be applied to measure uptake kinetics in field grown roots.

### 6.3.3 Nitrogen uptake by Eucalyptus nitens

#### *6.3.3.1 A preferable N source?*

Plants, including eucalypts, have been shown to have preferences for $NH_4^+$ or $NO_3^-$ as N sources (Moore and Keraitis, 1971; Chapin, 1988; Engels and Marschner, 1992; Lavoie et al., 1992; Shedley et al., 1995; Kronzucker et al., 1996). In conifers,

this preference is exhibited as reduced uptake rates of $NO_3^-$ with respect to $NH_4^+$ (Lavoie et al., 1992).

In the current depletion and MIFE experiments with *E. nitens,* rates of $NH_4^+$ uptake were consistently higher than those for $NO_3^-$ when presented at the same concentrations, irrespective of pH, N source, temperature and nutrient status. It appears reasonable to conclude that based on the preferential uptake of $NH_4^+$ by *E. nitens* , $NH_4^+$ is the preferred N source for *E. nitens*.

### *6.3.3.2 Applying knowledge of nitrogen uptake in very small solution cultured plants to trees in plantations*

These studies have been carried out on small (7-week old, depletion experiments) to very small (3-week old, MIFE experiments) solution cultured seedlings. For this reason it would be prudent to use caution when applying these results to describe N uptake by field grown trees. The root systems of the seedlings used here were white and non woody, which would correspond best to white, fine roots of field grown trees. In trees, fine roots that are constantly being replaced are thought to provide a large proportion of the nutrient and water uptake activity (Eissenstat and Van Rees, 1994). Older suberised or secondary thickened roots have a lower uptake activity than fine roots, but their contribution to total uptake may be high due to their large surface area (Eissenstat and Van Rees, 1994; Comerford et al., 1994). Although, accurate estimates of the N uptake by field grown roots would require a quantification of the uptake activity of older roots, the basic preference for $NH_4^+$ shown by solution cultured roots would be expected to be the same in older roots.

In terms of uptake rates, solution cultured *E. nitens* had a preference for $NH_4^+$ over $NO_3^-$ as an N source (Chapters 4 and 5), which might be related to the evolution of *E. nitens* on forest soils where $NH_4^+$ was the predominant N source. In view of this preference, N uptake by *E. nitens* in plantations will probably be maximised if the ratio of $NH_4^+$:$NO_3^-$ in soil solution is also maximised. Nitrification is limited and ammonium predominates in most cool, acidic soils with low rates of N mineralisation, such as those supporting native forests of south-eastern Australia <cite some of Adams and Attiwill, and Raison>. However, rates of nitrification increase after harvesting and preparation of the site for the next crop as rates of mineralisation

increase, rates of uptake decrease and $NH_4^+$ accumulates Smethurst et al., 1997. Although the $NH_4^+$:$NO_3^-$ ratio in soil solution probably decreases during this phase of the crop, it would increase again later as the crops demand for N increased and exceeded the rate of mineralisation, and as $NO_3^-$ concentrations decreased due to leaching, denitrification and uptake. If fertilisers were to be applied at this stage to increase N uptake and tree growth rates, maximum efficacy of applied N could be expected when $NH_4^+$ sources are used, e.g. ammonium sulphate or urea, as is currently practiced.

Temperate eucalypts in established plantations grow in association with both ecto- and endo-mycorrhiza (Grove et al., 1996). The N uptake characteristics of mycorrhizal roots may differ from that of non-mycorrhizal roots (George et al., 1995). In *E. diversicolor* the preference for ammonium over nitrate was lost in mycorrhizal roots (Dell et al., 1991). It was hypothesised that nitrate was assimilated by the mycorrhiza more efficiently than by the roots. It is unknown what effects mycorrhizal infection would have on the preference for $NH_4^+$ shown in experiments here.

# 7. Conclusions

Depletion and MIFE methods were used to investigate the uptake of $NH_4^+$ and $NO_3^-$ by solution cultured *E. nitens* seedlings in response to a range of environmental factors. As well as characterising N uptake by *E. nitens*, results highlighted differences in estimated uptake due to these two measurement approaches. Several conclusions can be drawn.

## *7.1 Nitrogen uptake by E. nitens*

The rate of $NH_4^+$ uptake was generally at least double that of $NO_3^-$ under all conditions (pH, temperature, N source, N status or growth rate) and with both methods of measurement (depletion and MIFE methods). Based on the kinetic parameters estimated from experiments using the MIFE method, *E. nitens* had both higher affinity, and higher uptake capacity, for $NH_4^+$ than for $NO_3^-$. These results, in conjunction with comparable studies on species from similar environments in the literature, suggest that $NH_4^+$ is the preferred but not exclusive N source for *E. nitens*.

## *7.2 The depletion method for measuring N uptake by plants*

Although the depletion method was initially developed as a simple method of estimating uptake kinetics, the method was difficult to apply and interpret. In future applications, care should be taken, to accurately collect data for a suitable range of concentrations, and in the subsequent analyses of these depletion curves to estimate parameters. A standardised approach to estimating parameters from depletion curves would aid comparisons between results of experiments using the depletion method.

The use of the depletion method to estimate kinetic parameters for N was suggested to be complicated by problems associated with efflux at higher concentrations. Removal of high concentration uptake data made it possible to estimate parameters, but this removal was subjective and led to large differences in parameter estimates. Hence, the apparent contribution of efflux to net N uptake made it difficult to interpret the results of N depletion experiments.

## *7.3 The MIFE method for measuring N uptake by plants*

The MIFE method was a good way of measuring N uptake by individual roots on small seedlings. The rapid nature and multi-ion attributes of the MIFE method make it a suitable method for studying the mechanisms and regulation of N uptake. These features also mean that it is more appropriate than the depletion method for studying many aspects of N uptake. Although the MIFE method did not provide estimates of $C_{min}$ , $K_m$ and $V_{max}$ for both $NH_4^+$and $NO_3^-$ were estimated in the low concentration (high affinity) range (< 200μM) and non-M-M uptake at higher concentrations was described for both $NH_4^+$ and $NO_3^-$.

# Bibliography

Abbes, C., Parent, L. E., Karam, A. and Isfan, D. (1995) Effect of $NH_4^+/NO_3^-$ ratios on growth and nitrogen uptake by onions. *Plant and Soil* 171: 289-296.

Adams, M. A. and Attiwill, P. M. (1986) Nutrient cycling and nitrogen mineralization in eucalypt forests of south-eastern Australia. I. Nutrient cycling and nitrogen turnover. *Plant and Soil* 92: 319-339.

Adams, M. A. and Attiwill, P. M. (1991) Nutrient balance in forests of northern Tasmania. I. Atmospheric inputs and within-stand cycles. *For. Ecol. Manage.* 44: 93-113.

Aguera, E., de la Haba, P, Fontes, A. G. and Maldonado, J. M. (1990) Nitrate and nitrite uptake and reduction by intact sunflower plants. *Planta* 182: 149-154.

Allen, S. and Smith, J. A. C. (1986) Ammonium nutrition in *Ricinis communis*: Its effect on plant growth and the chemical composition of the whole plant, xylem and phloem saps. *J. Exp. Bot.* 37: 1599-1610.

Asher, C. J. and Edwards, D. G. (1983) Modern solution culture techniques. *In* Encyclopedia of Plant Physiology, New Series, Inorganic plant nutrition, Vol. 15A. Edited by Lauchli, A. and Bieleski, R. L. pp. 94-119. Springer-Verlag, Berlin.

Aslam, M., Travis, R. L. and Huffaker, R. C. (1992) Comparative kinetics and reciprocal inhibition of nitrate and nitrite uptake in the roots of uninduced and induced barley (*Hordeum vulgare* L.) seedlings. *Plant Physiol.* 99: 1124-1133.

Aslam, M., Travis, R. L. and Huffaker, R. C. (1993) Comparative induction of nitrate and nitrite uptake and reduction systems by ambient nitrate and nitrite in intact roots of barley (*Hordeum vulgare* L.) seedlings. *Plant Physiol.* 102: 811-819.

Aslam, M., Travis, R. L. and Huffaker, R. C. (1994) Stimulation of nitrate and nitrite efflux by ammonium in barley (*Hordeum vulgare* L) seedlings. *Plant Physiol.* 106: 1293-1301.

Aslam, M., Travis, R. L. and Huffaker, R. C. (1995) Effect of pH and calcium on short-term $NO_3^-$ fluxes in roots of barley seedlings. *Plant Physiol.* 108: 727-734.

Aslam, M., Travis, R. L., Rains, D. W. and Huffaker, R. C. (1996) Effect of root perturbation and excision on nitrate influx and efflux in barley (*Hordeum vulgare*) seedlings. *Physiol. Plant.* 97: 425-432.

Ayling, S. A. (1993) The effect of ammonium ions on membrane potential and anion flux in roots of barley and tomato. *Plant Cell Environ.* 16: 297-303.

Barber, S. A. (1995) *Soil nutrient bioavailability: a mechanistic approach*, 2nd ed. Wiley, New York.

Bassirirad, H., Caldwell, M. M. and Bibrough, C. (1993) Effects of soil temperature and nitrogen status on kinetics of $^{15}NO_3^-$ uptake by roots of field-grown *Agropyron desertorum* (Fisch. ex Link) Schult. *New Phytol.* 123: 484-489.

Belton, P. S., Lee, R. B. and Ratcliffe, R. G. (1985) A $^{14}N$ nuclear magnetic resonance study of inorganic nitrogen metabolism in barley, maize and pea roots. *J. Exp. Bot.* 36: 190-210.

Bhat, K. K. S. (1981) Nutrient inflows into apple roots. I. $^{32}P$-orthophosphate uptake from solution by M.9 rootstocks and Worcester Permain seedlings. *Plant Cell Environ.* 4: 297-302.

Bhat, K. K. S. (1983) Nutrient inflows into apple roots. *Plant and Soil* 71: 371-380.

Bieleski, R. L. and Ferguson, I. B. (1983) Physiology and metabolism of phosphate and its compounds. *In* Encyclopedia of Plant Physiology, New Series, Inorganic plant nutrition, Vol. 15A. Edited by Läuchli, A. and Bieleski, R. L. pp. 422-449. Springer-Verlag, Berlin.

Bigot, J. and Boucaud, J. (1994) Low-temperature pre-treatment of the root system of *Brassica rapa* L. plants: Effects on the xylem sap exudation and on the nitrate absorption rate. *Plant Cell Environ.* 17: 721-729.

Birk, E. M. and Turner, J. (1991) Response of flooded gum (*E. grandis*) to intensive cultural treatment: biomass and nutrient content of eucalypt plantations and native forests. *For. Ecol. Manage.* 47: 1-28.

Bloom, A. J. (1985) Wild and cultivated barleys show similar affinities for mineral nitrogen. *Oecologia* 65: 555-557.

Bloom, A. J. (1988) Ammonium and nitrate as nitrogen sources for plant growth. *ISI Atlas of Science* 1: 55-59.

Bloom, A. J. (1989) Continuous and steady-state absorption by intact plants. *In* Applications of continuous and steady-state methods to root biology. Edited by Torrey, J. G. and Winship, L. J. pp. 147-163. Kluwer Academic Publishers, Dordrecht.

Bloom, A. J. and Epstein, E. (1984) Varietal differences in salt-induced respiration in barley. *Plant Sci. Lett.* 35: 1-3.

Bloom, A. J. and Finazzo, J. (1986) The influence of ammonium and chloride on potassium and nitrate absorption by barley roots depends on time of exposure and cultivar. *Plant Physiol.* 81: 67-69.

Bloom, A. J. and Caldwell, R. M. (1988) Root excision decreases nutrient absorption and gas fluxes. *Plant Physiol.* 87: 794-796.

Bloom, A. J. and Sukrapanna, S. S. (1990) Effects of exposure to ammonium and transplant shock upon the induction of nitrate absorption. *Plant Physiol.* 94: 85-90.

Boxman, A. W. and Roelofs, J. G. M. (1988) Some effects of nitrate versus ammonium nutrition on the nutrient fluxes in *Pinus sylvestris* seedlings. Effects of mycorrhizal infection. *Can. J. Bot.* 66: 1091-1097.

Bradley, P. M. and Morris, J. T. (1990) Influence of oxygen and sulphide concentration on nitrogen uptake kinetics in *Spartina alterniflora.* Ecology 71: 282-287.

Breteler, H. (1973) A comparison between ammonium and nitrate nutrition of young sugar-beet plants grown in nutrient solutions at constant acidity. 1. Production of dry matter, ionic balance and chemical composition. *Neth. J. Ag. Sci.* 21: 227-244.

Breteler, H. and Siegerist, M. (1984) Effect of ammonium on nitrate utilisation by roots of dwarf bean. *Plant Physiol.* 75: 1099-1103.

Burns, I. G. (1991) Short and long-term effects of a change in the spatial distribution of nitrate in the root zone on N uptake, growth and root development of young lettuce plants. *Plant Cell Environ.* 14: 21-33.

Carrodus, B. B. (1969) Studies on the metabolism of *Eucalyptus obliqua.* I. Inorganic sources of nitrogen, and the factors affecting the uptake of ammonia. *New Phytol.* 68: 1031-1039.

Chapin, F. S. (1988) Ecological aspects of plant mineral nutrition. *Advances in Plant Nutrition* 3: 161-191.

Chapin, F. S. (1995) Ecology - new cog in the nitrogen cycle. *Nature* 377: 199-200.

Chapin, F. S., Moilanen, L. and Kielland, K. (1993) Preferential use of organic nitrogen by a non-mycorrhizal artic sedge. *Nature* 361: 150-153.

Claassen, N. and Barber, S. A. (1974) A method for characterising the relation between nutrient concentration and flux into roots of intact plants. *Plant Physiol.* 54: 564-568.

Clarkson, D. T. (1985) Factors affecting mineral nutrient acquisition by plants. *Ann. Rev. Plant. Physiol.* 36: 77-116.

Clarkson, D. T. (1986) Regulation of the absorption and release of nitrate by plant cells: A review of current ideas and methodology. *In* Fundamental, ecological and Agricultural Aspects of Nitrogen Metabolism in Higher Plants. Edited by Lambers, H., Neeteson, J. J. and Stulen, I. pp. 3-27. Martinus Nijhoff Publishers, Dordrecht.

Clarkson, D. T. (1991) Root structure and sites of ion uptake. *In* Plant roots: the hidden half. Edited by Waisel, Y., Eshel, A. and Kafkafi, U. pp. 417-454. Marcel Dekker, New York.

Clarkson, D. T. and Warner, A. (1979) Relationship between root temperature and the transport of ammonium and nitrate ions by Italian and perennial ryegrass (*Lolium multiflorum* and *Lolium perenne*). *Plant Physiol.* 64: 557-561.

Clarkson, D. T. and Luttge, U. (1984) Mineral nutrition: vacuoles and tonoplasts. *Progress in Botany* 46: 56-67.

Clarkson, D. T., Hopper, M. J. and Jones, L. H. P. (1986) The effect of root temperature on the uptake of nitrogen and the relative size of the root system in *Lolium perenne*, I. Solutions containing both $NH_4^+$ and $NO_3^-$. *Plant Cell Environ.* 9: 535-545.

Clarkson, D. T. and Grignon, C. (1991) The phosphate transport system and its regulation in roots. *In* Phosphorous nutrition of grain legumes in the semi-arid tropics. Edited by Johansen, C., Lee, K. K. and Sahrawat, K. L. pp. 49-62. ICRISAT, Patancheru, India.

Clarkson, D. T., Jones, L. H. P. and Purves, J. V. (1992) Absorption of nitrate and ammonium ions by *Lolium perenne* from flowing solution cultures at low root temperatures. *Plant Cell Environ.* 15: 99-106.

Clarkson, D. T., Gojon, A., Saker, L. R., Wiersema, P. K., Purves, J. V., Tillard, P., Arnold, G. M., Paans, A. J. M., Vaalburg, W. and Stulen, I. (1996) Nitrate and ammonium influxes in soybean (*Glycine max*) roots -direct comparison of $^{13}N$ and $^{15}N$ tracing. *Plant Cell Environ.* 19: 859-868.

Clement, C. R., Hopper, M. J. and Jones, L. H. P. (1978a) The uptake of nitrate by *Lolium perenne* from flowing culture solution. I. Effect of $NO_3^-$ concentration. *J. Exp. Bot.* 29: 453-464.

Clement, C. R., Hopper, M. J., Jones, L. H. P. and Leafe, E. L. (1978b) The uptake of nitrate by *Lolium perenne* from flowing culture solution. II. Effect of light, defoliation, and relationship to $CO_2$ flux. *J. Exp. Bot.* 29: 1173-1183.

Cogliatti, D. and Clarkson, D. T. (1983) Physiological changes in, and phosphate uptake by potato plants during development of, and recovery from phosphate deficiency. *Physiol. Plant.* 58: 287-294.

Comerford, N. B., Smethurst, P. J. and Escamilla, J. A. (1994) Nutrient uptake by woody root systems. *N. Z. J. For. Sci.* 24: 195-212.

Cooper and Clarkson, D. T. (1989) Cycling of amino-nitrogen and other nutrients between shoots and roots in cereals-A possible mechanism for integrating shoot and root in the regulation of nutrient uptake. *J. Exp. Bot.* 40: 753-762.

Cromer, R. N., Cameron, D., Cameron, J. N., Flinn, D. W., Neilson, W., Raupach, M., Snowdon, P. and Waring, H. (1981) Response of eucalypt species to fertilizer applied soon after planting at several sites. *Australian Forestry* 44: 3-13.

Cromer, R. N. and Jarvis, P. G. (1990) Growth and biomass partitioning in *Eucalyptus grandis* seedlings in response to nitrogen supply. *Aust. J. Plant. Physiol.* 17: 503-515.

Crossett, A. N. and Campbell, D. J. (1975) The effects of ethylene in the root environment upon the development of barley. *Plant and Soil* 42: 453-464.

Cruz, C., Lips, S. H. and Martinsloucao, M. A. (1993) Uptake of ammonium and nitrate by carob (*Ceratonia siliqua*) as affected by root temperature and inhibitors. *Physiol. Plant.* 89: 532-543.

Davies, J. L., Ed. (1965) *Atlas of Tasmania*. Lands and Survey Department, Hobart.

Deane-Drummond, C. E. (1986) Nitrate uptake into *Pisum sativum* L. cv. Feltham First seedlings: Commonality with nitrate uptake into *Chara corallina* and *Hordeum vulgare* through a substrate cycling model. *Plant Cell Environ.* 9: 41-48.

Deane-Drummond, C. E. and Glass, A. D. M. (1982) Nitrate uptake into barley (*Hordeum vulgare*) plants. A new approach using $^{36}ClO_3^-$ as and analog for $NO_3^-$ *Plant Physiol.* 70: 50-54.

Deane-Drummond, C. E. and Glass, A. D. M. (1983) Short term studies of nitrate uptake into barley plants using ion-specific electrodes and $^{36}ClO_3^-$. II Regulation of $NO_3^-$ efflux by $NH_4^+$. *Plant Physiol.* 73: 105-110.

Delhon, P., Gojon, A., Tillard, P. and Passama, L. (1995) Diurnal regulation of $NO_3^-$ uptake in soybean plants .1. Changes in $NO_3^-$ influx, efflux, and N utilization in the plant during the day night cycle. *J. Exp. Bot.* 46: 1585-1594.

Delhon, P., Gojon, A., Tillard, P. and Passama, L. (1996) Diurnal regulation of $NO_3^-$ uptake in soybean plants .4. Dependence on current photosynthesis and sugar availability to the roots. *J. Exp. Bot.* 47: 893-900.

Dell, B., Grove, T. S. and Malajczuk, N. (1991) Effect of ammonium and nitrate on growth and nitrogen accumulation in ectomycorrhizal and uninoculated seedlings of *Euclayptus diversicolor*. Third Australian Forest Soils and Nutrition Conference Proceedings, pp. 210.

Devienne, F., Mary, B. and Lamaze, T. (1994) Nitrate transport in intact wheat roots. 1. Estimation of cellular fluxes and $NO_3^-$ distribution using compartmental analysis from data of $^{15}NO_3^-$ efflux. *J. Exp. Bot.* 45: 667-676.

Doddema, H. and Telkamp, G. R. (1979) Uptake of nitrate by mutants of *Arabidopsis thaliana*, disturbed in uptake or reduction of nitrate. II. Kinetics. *Physiol. Plant.* 45: 332-338.

Doddema, H. and Otten, H. (1979) Uptake of nitrate by mutants of *Aribidopsis thaliana*, disturbed in uptake or reduction of nitrate: III. Regulation. *Physiol. Plant.* 45: 339-346.

Drew, M. C., Saker, L. R., Barber, S. A. and Jenkins, W. (1984) Changes in the kinetics of phosphate and potassium absorption in nutrient-deficient barley roots measured by a solution-depletion technique. *Planta* 160: 490-499.

Drew, M. C. and Saker, L. R. (1984) Uptake and long-distance transport of phosphate, potassium and chloride in relation to internal ion concentrations in barley: evidence for non-allosteric regulation. *Planta* 160: 500-507.

Edwards, J. H. and Barber, S. A. (1976) Nitrogen uptake characteristics of corn roots at low N concentrations as influenced by plant age. *Agron. J.* 68: 17-19.

Eissenstat, D. M. and Van Rees, K. J. C. (1994) The growth and function of pine roots. *Ecological Bulletins* 43: 76-91.

Engels, C. and Marschner, H. (1992) Root to shoot translocation of macronutrients in relation to shoot demand in maize (*Zea mays* L.) grown at different root zone temperatures. *Z. Pflanzenernähr. Bodenk.* 155: 121-128.

Engels, C. and Marschner, H. (1995) Plant uptake and utlization of nitrogen. *In* Nitrogen fertilization in the environment. Edited by Bacon, P. E. pp. 41-81. Marcel Dekker, New York.

Epstein, E. (1972) *Mineral Nutrition of Higher Plants: Principles and Perspectives.* John Wiley, New York.

Epstein, E. and Hagan, C. E. (1952) A kinetic study of the absorption of alkali cations by barley roots. *Plant Physiol.* 27: 457-474.

Epstein, E., Schmid, W. E. and Rains, D. W. (1963) Significance and technique of short-term experiments on solute absorption by plant tissue. *Plant and Cell Physiology* 4: 79-84.

Felle, H. (1987) Proton transport and pH control in *Sinapis alba* root hairs: A study carried out with double-barrelled pH micro-electrodes. *J. Exp. Bot.* 38: 340-354.

Felle, H. (1988) Short-term pH regulation in plants. *Physiol. Plant.* 74: 583-591.

Fernandes, M. S. and Rossiello, R. O. P. (1995) Mineral nitrogen in plant physiology and plant nutrition. *Critical Reviews in Plant Sciences* 14: 111-148.

Fried, M., Zsoldos, F., Vose, P. B. and Shatokhin, I. L. (1965) Characterising the $NO_3^-$ and $NH_4^+$ uptake process of rice roots by use of $^{15}N$ labelled $NH_4NO_3$. *Physiol. Plant.* 18: 313-319.

George, E., Marschner, H. and Jakobsen, I. (1995) Role of arbuscular mycorrhizal fungi in uptake of phosphorus and nitrogen from soil. *Critical Reviews in Biotechnology* 15: 257-270.

Givan, C. V. (1979) Metabolic detoxification of ammonia in tissues of higher plants. *Phytochemistry* 18: 375-382.

Glass, A. D. M. (1978) Influence of excision and ageing upon $K^+$ influx into barley roots. Recovery or enhancement? *Plant Physiol.* 61: 481-483.

Glass, A. D. M. (1988) Nitrogen uptake by plant roots. *CRC Atlas of Science. Animal and plant sciences,* 1: 151-156.

Glass, A. D. M. (1989) *Plant nutrition: an introduction to current concepts.* Jones and Bartlett Publishers, Boston.

Glass, A. D. M. (1990) Ion absorption and utilization: The cellular level. *In* Crops as enhancers of nutrient use. Edited by Baligar, V. C. and Duncan, R. R. pp. 37-64. Academic Press, New York.

Glass, A. D. M., Thompson, R. G. and Bordeleau, L. (1985) Regulation of $NO_3^-$ influx in barley. Studies using $^{13}NO_3^-$. *Plant Physiol.* 77: 379-381.

Glass, A. D. M., Siddiqi, M. Y., Ruth, T. J. and Rufty, T. W. (1990) Studies on the uptake of nitrate in barley. 2. Energetics. *Plant Physiol.* 93: 1585-1589.

Glass, A. D. M., Schaff, J. E. and Kochian, L. V. (1992) Studies of the uptake of nitrate in barley. 4. Electrophysiology. *Plant Physiol.* 99: 456-463.

Goyal, S. S. and Huffaker, R. C. (1986) The uptake of $NO_3^-$, $NO_2^-$ and $NH_4^+$ by intact wheat (*Triticum aestivum*) seedlings. I. Induction and kinetics of transport systems. *Plant Physiol.* 82: 1051-1056.

Gronewald, J. W. and Hanson, J. B. (1980) Sensitivity of the proton and ion transport mechanisms of corn roots to injury. *Plant Sci. Lett.* 18: 143-150.

Grove, T. S., Malajczuk, N. and Hardy, G. E. S. (1996) The effect of soil pH on the ability of ectomycorrhizal fungi to increase the growth of *Eucalyptus globulus* Labill. *Plant and Soil* 178: 209-214.

Gutschick, V. P. (1993) Nutrient-limited growth rates: Roles of nutrient-use efficiency and of adaptations to increase uptake rate. *J. Exp. Bot.* 44: 41-51.

Haynes, R. J. and Goh, K. M. (1978) Ammonium and nitrate nutrition of plants. *Biol. Rev.* 53: 465-510.

Henriksen, G. H., Bloom, A. J. and Spanswick, R. M. (1990) Measurement of net fluxes of ammonium and nitrate at the surface of barley roots using ion-selective microelectrodes. *Plant Physiol.* 93: 271-280.

Henriksen, G. H., Raj Raman, D., Walker, L. P. and Spanswick, R. M. (1992) Measurement of net fluxes of ammonium and nitrate at the surface of barley roots using ion-sensitive microelectrodes. II. Patterns of uptake along the root axis and evaluation of the microelectrode flux estimation technique. *Plant Physiol.* 99: 734-747.

Henriksen, G. H. and Spanswick, R. M. (1993) Investigation of the apparent induction of nitrate uptake in barley (*Hordeum vulgare* L.) using $NO_3^-$-selective microelectrodes: Modulation of coarse regulation of $NO_3^-$ uptake by exogenous application of downstream metabolites in the $NO_3^-$ assimilatory pathway. *Plant Physiol.* 103: 885-892.

Hewitt, E. J. (1966) *Sand and water culture methods used in the study of plant nutrition*, Techical Communication No.22 (Revised 2nd Edition), Commonwealth Bureau of Horticulture and Plantation Crops, East Malling, Commonwealth Agricultural Bureaux, Farnham Royal, England.

Hingston, F. J. and Jones, M. S. (1985) Changes in the composition of soil solutions resulting from application of fertilizer to Jarrah forest in south-western Australia. *Aust. J. For. Res.* 15: 293-308.

Huang, Z.-Z., Yan, Y., Jalil, A., Norlyn, J. D. and Epstein, E. (1992) Short-term experiments on ion transport by seedlings and excised roots. *Plant Physiol.* 100: 1914-1920.

Ingestad, T. and Lund, A.-B. (1986) Theory and techniques for steady state mineral nutrition and growth of plants. *Scand. J. For. Res.* 1: 439-453.

Ingestad, T. and Ågren, G. I. (1992) Theories and methods on plant nutrition and growth. *Physiol. Plant.* 84: 177-184.

Jackson, R. B., Manwaring, J. H. and Caldwell, M. M. (1990) Rapid physiological adjustment of roots to localised soil enrichment. *Nature* 344: 58-60.

Jackson, R. B. and Caldwell, M. M. (1991) Kinetic responses of *Pseudoroegneria* roots to localized soil enrichment. *Plant and Soil* 138: 231-238.

Jackson, W. A., Flesher, D. and Hageman, R. H. (1973) Nitrate uptake by dark grown corn seedlings: Some characteristics of the apparent induction. *Plant Physiol.* 51: 120-127.

Jackson, W. A. and Volk, R. J. (1992) Nitrate and ammmonium uptake by maize: adaption during relief from nitrogen suppression. *New Phytol.* 122: 439-446.

Jaffe, L. F. and Nuccitelli, R. (1974) An ultrasensitive vibrating probe for measuring steady extracellular currents. *J. Cell Biol.* 63: 614-628.

Jones, D. L. and Darrah, P. R. (1994) Amino-acid influx at the soil-root interface of *Zea mays* L. and its implications in the rhizosphere. *Plant and Soil* 163: 1-12.

Jones, D. L., Shaff, J. E. and Kochian, L. V. (1995) Role of calcium and other ions in directing root hair tip growth in *Limnobium stoliferum*. *Planta* 197: 672-680.

Jones, H. E., Quarmby, C. and Harrison, A. F. (1991) A root bioassay test for nitrogen deficiency in forest trees. *For. Ecol. Manage.* 42: 267-282.

Joseph, R. A., Hai, T. V. and Lambert, J. (1975) Multiphasic uptake of ammonium by soybean roots. *Physiol. Plant.* 34: 321-325.

Judd, T. S., Bennett, L. T., Weston, C. J., Attiwill, P. M. and Whiteman, P. H. (1996) The response of growth and foliar nutrients to fertilizers in young *Eucalyptus globulus* (Labill.) plantations in Gippsland, southeastern Australia. *For. Ecol. Manage.* 82: 87-101.

Kamminga-van Wijk, C. and Prins, H. B. A. (1993) The kinetics of $NH_4^+$ and $NO_3^-$ uptake by Douglas Fir from single N-solutions and from solutions containing both $NH_4^+$ and $NO_3^-$. *Plant and Soil* 151: 91-96.

Kennedy, C. D. and Gonsalves, F. A. N. (1988) $H^+$ efflux and trans-root potential measured while increasing the temperature of solutions bathing excised roots of *Zea mays*. *J. Exp. Bot.* 39: 37-49.

King, B. J., Siddiqi, M. Y. and Glass, A. D. M. (1992) Studies of the uptake of nitrate in barley. 5. Estimation of root cytoplasmic nitrate concentration using nitrate reductase activity-implications for nitrate influx. *Plant Physiol.* 99: 1582-1589.

King, B. J., Siddiqi, M. Y., Ruth, T. J., Warner, R. L. and Glass, A. D. M. (1993) Feedback regulation of nitrate influx in barley roots by nitrate, nitrite, and ammonium. *Plant Physiol.* 102: 1279-1286.

Kleiner, D. (1981) The transport of $NH_3$ and $NH_4^+$ across biological membranes. *Biochem. Biophys. Acta* 639: 41-52.

Knoepp, J. D., Turner, D. P. and Tingey, D. T. (1993) Effects of ammonium and nitrate on nutrient uptake and activity of nitrogen assimilating enzymes in Western Hemlock. *For. Ecol. Manage.* 59: 179-191.

Kochian, L. V., Shaff, J. E., Kuhtreiber, W. M., Jaffe, L. F. and Lucas, W. J. (1992) Use of an extracellular, ion-selective, vibrating microelectrode system for the quantification of $K^+$, $H^+$, and $Ca^{++}$ fluxes in maize roots and maize suspension cells. *Planta* 188: 601-610.

Kronzucker, H. J., Siddiqi, M. Y. and Glass, A. D. M. (1995a) Compartmentation and flux characteristics of nitrate in spruce. *Planta* 196: 674-682.

Kronzucker, H. J., Glass, A. D. M. and Siddiqi, M. Y. (1995b) Nitrate induction in spruce - an approach using compartmental analysis. *Planta* 196: 683-690.

Kronzucker, H. J., Siddiqi, M. Y. and Glass, A. D. M. (1995c) Compartmentation and flux characteristics of ammonium in spruce. *Planta* 196: 691-698.

Kronzucker, H. J., Siddiqi, M. Y. and Glass, A. D. M. (1995d) Kinetics of $NO_3^-$ influx in spruce. *Plant Physiol.* 109: 319-326.

Kronzucker, H. J., Siddiqi, M. Y. and Glass, A. D. M. (1996) Kinetics of $NH_4^+$ influx in spruce. *Plant Physiol.* 110: 773-779.

Kuhtreiber, W. M. and Jaffe, L. F. (1990) Detection of extracellular calcium gradients with a calcium-specific vibrating electrode. *J. Cell Biol.* 110: 1565-1573.

Laine, P., Ourry, A., Macduff, J., Boucard, J. and Salette, J. (1993) Kinetic parameters of nitrate uptake by different catch crop species: effects of low temperatures or previous nitrate starvation. *Physiol. Plant.* 88: 85-92.

Larsson, C.-M. (1994) Responses of the nitrate uptake system to external nitrate availability: A whole plant perspective. *In* A Whole Plant Perspective on Carbon-Nitrogen Interactions. Edited by Roy, J. and Garnier, E. pp. 31-45. SPD Academic Publishing, The Hague.

Larsson, C.-M., Larsson, M., Purves, J. V. and Clarkson, D. T. (1991) Translocation and cycling through roots of recently absorbed nitrogen and sulfur in wheat (*Triticum aestivum*) during vegetative and generative growth. *Physiol. Plant.* 82: 345-352.

Larsson, C. M., Mattsson, M., Duarte, P., Samuelson, M., Ohlen, E., Oscarson, P., Ingemarsson, B., Larsson, M. and Lundborg, T. (1992) Uptake and assimilation of nitrate under nitrogen limitation. *In* Nitrogen metabolism of plants. Edited by Mengel, K. and Pilbeam, D. J. pp. 71-90. Oxford University Press, Oxford.

Lauter, F. R., Ninnemann, O., Bucher, M., Riesmeier, J. W. and Frommer, W. B. (1996) Preferential expression of an ammonium transporter and of two putative nitrate transporters in root hairs of tomato. *Proc. Nat. Acad. Sci. USA* 93: 8139-8144.

Lavoie, N., Vezina, L. P. and Margolis, H. A. (1992) Absorption and assimilation of nitrate and ammonium ions by Jack pine seedlings. *Tree Physiol.* 11: 171-183.

Lazof, D. B., Rufty, T. W. and Redinbaugh, M. G. (1992) Localization of nitrate absorption and translocation within morphological regions of the corn root. *Plant Physiol.* 100: 1251-1258.

Le Bot, J. and Kirkby, E. A. (1992) Diurnal uptake of nitrate and potassium during the vegetative growth of tomato plants. *J. Plant Nutr.* 15: 247-264.

Lee, R. B. (1982) Selectivity and kinetics of ion uptake by barley plants following nutrient deficiency. *Ann. Bot.* 50: 429-449.

Lee, R. B. (1993) Control of net uptake of nutrients by regulation of influx in barley plants recovering from nutrient deficiency. *Ann. Bot.* 72: 223-230.

Lee, R. B. and Clarkson, D. T. (1986) Nitrogen-13 studies of nitrate fluxes in barley roots. I. Compartmental analysis from measurements of $^{13}N$ efflux. *J. Exp. Bot.* 37: 1753-1767.

Lee, R. B. and Drew, M. C. (1986) Nitrogen-13 studies of nitrate fluxes in barley roots. II. Effect of plant N-status on the kinetic parameters of nitrate influx. *J. Exp. Bot.* 37: 1768-1779.

Lee, R. B. and Rudge, K. A. (1986) Effects of nitrogen deficiency on the absorption of nitrate and ammonium by barley plants. *Ann. Bot.* 57: 471-486.

Lee, R. B. and Drew, M. C. (1989) Rapid, reversible inhibition of nitrate influx in barley by ammonium. *J. Exp. Bot.* 40: 741-752.

Lee, R. B. and Ratcliffe, R. G. (1991) Observations on the subcellular distribution of the ammonium ion in maize root tissue using in vivo $^{14}$N-nuclear magnetic resonance spectroscopy. *Planta* 183: 359-367.

Leigh, R. A. and Wyn Jones, R. G. (1986) Cellular compartmentation in plant nutrition: the selective cytoplasm and the promiscuous vacuole. *Advances in Plant Nutrition* 2: 249-279.

Lowther, J. R. (1980) Use of a single acid-hydrogen peroxide digest for the analysis of *Pinus radiata* needles. *Commun. Soil. Sci. Plant. Anal.* 11: 175-188.

Lucas, W. J. and Kochian, L. V. (1986) Ion transport processes in corn roots: An approach utilizing microelectrode techniques. *In* Advanced agricultural instrumentation. Edited by Gensler, W. G. pp. 402-425. Martinus Nijhoff Publishers, Dordrecht Boston Lancaster.

Lycklama, J. C. (1963) The absorption of ammonium and nitrate by perennial rye-grass. *Acta Bot. Neerl.* 12: 361-423.

Maathuis, F. J. M. and Sanders, D. (1996) Mechanisms of potassium absorption by higher plant roots. *Physiol. Plant.* 96: 158-168.

Macduff, J. H. and Hopper, M. J. (1986) Effects of root temperature on uptake of nitrate and ammonium ions by barley grown in flowing solution culture. *Plant and Soil* 91: 303-306.

Macduff, J. H., Hopper, M. J. and Wild, A. (1987) The effect of root temperature on growth and uptake of ammonium and nitrate by *Brassica napus* L. cv. Bien venu in flowing solution culture. II. Uptake from solutions containing $NH_4NO_3$. *J. Exp. Bot.* 38: 53-66.

Macduff, J. H. and Wild, A. (1989) Interactions between root temperature and nitrogen deficiency influence preferential uptake of $NH_4^+$ and $NO_3^-$ by oilseed rape. *J. Exp. Bot.* 40: 195-206.

Macduff, J. H. and Jackson, S. B. (1991) Growth and preferences for ammonium and nitrate uptake by barley in relation to root temperature. *J. Exp. Bot.* 42: 521-530.

Macduff, J. H. and Jackson, S. B. (1992) Influx and efflux of nitrate and ammonium in Italian rye grass and white clover roots: comparisons between effects of darkness and defoliation. *J. Exp. Bot.* 43: 525-535.

Macduff, J. H., Jarvis, S. C. and Cockburn, J. E. (1994) Acclimation of $NO_3^-$ fluxes to low root temperature by *Brassica napus* in relation to $NO_3^-$ supply. *J. Exp. Bot.* 45: 1045-1056.

MacFarlane, J. J. and Smith, F. A. (1982) Uptake of methylamine by *Ulva rigida*: transport of cations and diffusion of free base. *J. Exp. Bot.* 33: 195-207.

Mäck, G. and Tischner, R. (1994) Constitutive and inducible net $NH_4^+$ uptake of barley (*Hordeum vulgare* L.) seedlings. *J. Plant. Physiol.* 144: 351-357.

Macklon, A. E. S., Ron, M. M. and Sim, A. (1990) Cortical cell fluxes of ammonium and nitrate in excised root segments of *Allium cepa* L.; studies using $^{15}N$. *J. Exp. Bot.* 224: 359-370.

MacKown, C. T., Jackson, W. A. and Volk, R. J. (1982) Restricted nitrate influx an reduction in corn seedlings exposed to ammonium. *Plant Physiol.* 69: 353-359.

Marcus-Wyner, L. (1983) Influence of ambient acidity on the absorption of $NO_3^-$ and $NH_4^+$ by tomato plants. *J. Plant Nutr.* 6: 657-666.

Marschner, H. (1995) *Mineral Nutrition of Higher Plants*, 2nd ed. Academic Press, London.

Marschner, H., Haussling, M. and George, E. (1991) Ammonium and nitrate uptake rates and rhizosphere pH in non-mycorrhizal roots of Norway spruce (*Picea abies* (L.) Karst). *Trees* 5: 14-21.

Mattsson, M., Johansson, E., Lundborg, T., Larsson, M. and Larsson, C.-M. (1991) Nitrogen utilization in N-limited barley during vegetative and generative

growth. I. Growth and nitrate uptake kinetics in vegetative cultures grown at different relative addition rates of nitrate-N. *J. Exp. Bot.* 42: 197-205.

McClure, P. R., Kochian, L. V., Spanswick, R. M. and Shaff, J. E. (1990) Evidence for cotransport of nitrate and protons in maize roots. II. Measurement of $NO_3^-$ and $H^+$ fluxes with ion-sensitive microelectrodes. *Plant Physiol.* 93: 290-294.

McLeod, S. (1992) Determination of total soil and plant nitrogen using a micro-distillation unit in a continuos flow analyser. *Anal. Chim. Acta.* 266: 113-117.

Medeiros, C. A. B., Clark, R. B. and Ellis, J. R. (1993) Effects of MES [2(N-morpholino)-ethanesulfonic acid] and pH on mineral nutrient uptake by mycorrhizal and nonmycorrhizal maize. *J. Plant Nutr.* 16: 2255-2272.

Meharg, A. A. and Blatt, M. R. (1995) $NO_3^-$ transport across the plasma membrane of *Arabidopsis thaliana* root hairs - kinetic control by pH and membrane voltage. *J. Membrane Biol.* 145: 49-66.

Miller, A. J. and Smith, S. J. (1996) Nitrate transport and compartmentation in cereal root cells. *J. Exp. Bot.* 47: 843-854.

Miller, A. L., Shand, E. and Gow, N. A. R. (1988) Ion currents associated with root tips, emerging laterals. *Plant Cell Environ.* 11: 21-25.

Minotti, P. L., Williams, D. C. and Jackson, W. A. (1969) Nitrate uptake by wheat as influenced by ammonium and other cations. *Crop Sci.* 9: 9-14.

Moore, C. W. E. and Keraitis, K. (1971) Effect of nitrogen source on growth of eucalypts in sand culture. *Aust. J. Bot.* 19: 125-141.

Morgan, M. A. and Jackson, W. A. (1988a) Inward and outward movement of ammonium in root systems: transient responses during recovery from nitrogen deprivation in presence of ammonium. *J. Exp. Bot.* 39: 179-191.

Morgan, M. A. and Jackson, W. A. (1988b) Suppression of ammonium uptake by nitrogen supply and its relief during nitrogen limitation. *Physiol. Plant.* 73: 38-45.

Morgan, M. A. and Jackson, W. A. (1989) Reciprocal ammonium transport into and out of plant roots: modifications by plant nitrogen status and elevated root ammonium concentration. *J. Exp. Bot.* 40: 207-214.

Morris, J. T. (1980) The nitrogen uptake kinetics of *Spartina alternifolia* in culture. *Ecology* 61: 1114-1121.

Muller, B. and Touraine, B. (1992) Inhibition of $NO_3^-$ uptake by various phloem-translocated amino acids in soybean seedlings. *J. Exp. Bot.* 43: 617-623.

Muller, B., Tillard, P. and Touraine, B. (1995) Nitrate fluxes in soybean seedling roots and their response to amino acids - an approach using $^{15}N$. *Plant Cell Environ.* 18: 1267-1279.

Mullins, G. L. and Edwards, J. H. (1988) A comparison of two methods for measuring potassium influx kinetics by intact corn seedlings. *J. Plant Nutr.* 12: 485-496.

Munn, D. A. and Jackson, W. A. (1978) Nitrate and ammonium uptake by rooted cuttings of sweet potato. *Agron. J.* 70: 312-316.

Newman, I. A., Kochian, L. V., Grusak, M. A. and Lucas, W. J. (1987) Fluxes of $H^+$ and $K^+$ in corn roots. Characterization and stoichiometries using ion-selective microelectrodes. *Plant Physiol.* 84: 1177-1184.

Neyra, C. A. and Hageman, R. H. (1975) Nitrate uptake and induction of nitrate reductase in excised corn roots. *Plant Physiol.* 56: 692-695.

Nicholas, J. C. and Harper, J. E. (1993) Effect of MES [2(N-morpholino)ethanesulphonic acid] and Amberlite IRC-50 resin on nutrient pH control and soybean growth. *J. Plant Nutr.* 16: 895-909.

Nielson, N. E. and Barber, S. A. (1978) Differences among genotypes of corn in the kinetics of P uptake. *Agron. J.* 70: 695-698.

Ninnemann, O., Jauniaux, J.-C. and Frommer, W. B. (1994) Identification of a high affinity $NH_4^+$ transporter from plants. *Embo J.* 13: 3464-3471.

Nobel, P. S. (1983) *Biophysical plant physiology and ecology.* W.H. Freeman, San Francisco.

Oaks, A. (1994) Primary nitrogen assimilation in higher plants and its regulation. *Can. J. Bot.* 72: 739-750.

Oates, K. and Barber, S. A. (1987) NUTRIENT UPTAKE: A microcomputer program to predict nutrient absorption from soil by roots. *J. Agron. Educ.* 16: 65-68.

Oscarson, P., Ingemarsson, B., af Ugglass, M. and Larsson, C.-M. (1987) Short-term studies of $NO_3^-$ uptake in *Pisum* using $^{13}NO_3^-$. *Planta* 170: 550-555.

Oscarson, P., Ingemarsson, B. and Larsson, C.-M. (1989) Growth and nitrate uptake properties of plants grown at different relative rates of nitrogen supply. II. Activity and affinity of the nitrate uptake system in *Pisum* and *Lemna* in relation to nitrogen availability and nitrogen demand. *Plant Cell Environ.* 12: 787-794.

Pace, G. M. and Mcclure, P. R. (1986) Comparison of nitrate uptake kinetic parameters across maize inbred lines. *J. Plant Nutr.* 9: 1095-1111.

Padgett, P. E. and Leonard, R. T. (1993) Contamination of ammonium-based nutrient solutions by nitrifying organisms and the conversion of ammonium to nitrate. *Plant Physiol.* 101: 141-146.

Peuke, A. D. and Tischner, R. (1991) Nitrate uptake and utilisation of aseptically cultivated Spruce seedlings, *Picea abies* (L.) Karst. *J. Exp. Bot.* 42: 723-728.

Peuke, A. D., Glaab, J., Kaiser, W. M. and Jeschke, W. D. (1996) The uptake and flow of C, N and ions between roots and shoots in *Ricinus communis* L. 4. Flow and metabolism of inorganic nitrogen and malate depending on nitrogen nutrition and salt treatment. *J. Exp. Bot.* 47: 377-385.

Pilbeam, D. J. and Kirkby, E. A. (1992) Some aspects of the utilization of nitrate and ammonium by plants. *In* Nitrogen metabolism of plants. Edited by Mengel, K. and Pilbeam, D. J. pp. 55-70. Oxford University Press, Oxford.

Popp, M. (1993) Ecological aspects of nitrogen nutrition. *Progress in Botany*, 54: 448-460.

Raman, D. R., Spanswick, R. M. and Walker, L. P. (1995) High temporal resolution measurement of nitrate uptake from flowing solutions. *Biores. Technol.* 53: 113-123.

Raman, D. R., Spanswick, R. M. and Walker, L. P. (1995) The kinetics of nitrate uptake from flowing solutions by rice -influence of pretreatment and light. *Biores. Technol.* 53: 125-132.

Rao, K. P. and Rains, D. W. (1976) Nitrate absorbtion by barley. II. Kinetics and energetics. *Plant Physiol.* 57: 59-62.

Raven, J. A. and Smith, F. A. (1976) Nitrogen assimilation and transport in vascular land plants in relation to intracellular pH regulation. *New Phytol.* 76: 415-431.

Rideout, J. W. and Raper, C. D. (1994) Diurnal changes in net uptake rate of nitrate are associated with changes in estimated export of carbohydrates to roots. *Int. J. Plant Sci.* 155: 173-179.

Ritchie, R. J. (1987) The permeability of ammonia, methylamine and ethylamine in the charophyte *Chara corallina*. *J. Exp. Bot.* 38: 67-76.

Ritchie, R. J. and Pravan, T. (1996) A simulation study on designing experiments to measure the $K_m$ of Michaelis-Menten kinetics curves. *J. Theor. Biol.* 178: 239-254.

Roberts, J. K. M. and Pang, M. K. L. (1992) Estimation of ammonium ion distribution between cytoplasm and vacuole using nuclear magnetic resonance spectroscopy. *Plant Physiol.* 100: 1571-1574.

Robinson, D. (1994) The responses of plants to non-uniform supplies of nutrients. *New Phytol.* 127: 635-674.

Robinson, R. A. and Stokes, R. H. (1959) *Electrolyte solutions*. Butterworths, London.

Rorison, I. H., Peterkin, J. H. and Clarkson, D. T. (1983) Nitrogen source, temperature and the growth of herbaceous plants. *In* Nitrogen as an ecological factor. Edited by Lee, J. A., McNeil, S. and Rorison, I. H. pp. 189-209. Blackwell, Oxford.

Runge, M. (1981) Physiology and ecology of nitrogen nutrition. *In* Physiological Plant Ecology III, Responses to the Chemical and Biological Enviroment, Vol. 12C. Edited by Lange, O. L., Nobel, P. S., Osmond, C. B. and Zeigler, H. pp. 163-200. Springer-Verlag, Berlin.

Ryan, P. R., Newman, I. A. and Sheilds, B. (1990) Ion fluxes in corn roots measured by microelectrodes with ion specific liquid membranes. *J. Memb. Sci.* 53: 59-69.

Ryan, P. R. and Walker, N. A. (1994) The regulation of ammonia uptake in *Chara australis*. *J. Exp. Bot.* 45: 1057-1067.

Samuelson, M. E., Ohlen, E., Lind, H. and Larsson, C.-M. (1995) Nitrate regulation of nitrate uptake and nitrate reductase expression in barley grown at different nitrate/ammonium ratios at constant relative nitrogen addition rate. *Physiol. Plant.* 94: 254-260.

Sands, P. J. and Smethurst, P. J. (1995) Modelling nitrogen uptake in Ingestad units using Michaelis-Menten kinetics. *Aust. J. Plant. Physiol.* 22: 823-831.

Scaife, A. (1989) A pump/leak/buffer model for plant nitrate uptake. *Plant and Soil* 114: 139-141.

Scaife, A. and Schloemer, S. (1994) The diurnal pattern of nitrate uptake and reduction by spinach (*Spinacia oleracea* L.). *Ann. Bot.* 73: 337-343.

Scheromm, P. and Plassard, C. (1988) Nitrogen metabolism of non-mycorrhizal pine (*Pinus pinaster*) grown on nitrate or ammonium. *Plant. Physiol. Biochem.* 26: 261-269.

Schlee, J. and Komor, E. (1986) Ammonium uptake by *Chlorella*. *Planta* 168: 232-238.

Schönau, A. P. G. and Herbert, M. A. (1989) Fertilizing eucalypts at plantation establishment. *For. Ecol. Manage.* 29: 221-244.

Schulze, E. D., Chapin, F. S. and Gebauer, G. (1994) Nitrogen nutrition and isotope differences among life forms at the northern treeline of alaska. *Oecologia* 100: 406-412.

Shabala, S. N., Newman, I. A. and Morris, J. (1997) Oscillations in hydrogen and calcium ion fluxes around the elongation region of corn roots and effects of external pH. *Plant Physiol.* In press.

Shedley, E., Dell, B. and Grove, T. (1995) Diagnosis of nitrogen deficiency and toxicity of *Eucalyptus globulus* seedlings by foliar analysis. *Plant and Soil* 177: 183-189.

Siddiqi, M. Y., Glass, A. D. M., Ruth, T. J. and Fernando, M. (1989) Studies of the regulation of nitrate influx by barley seedlings using $^{13}NO_3^-$. *Plant Physiol.* 90: 806-813.

Siddiqi, M. Y., Glass, A. D. M., Ruth, T. J. and Rufty, T. W. (1990) Studies on the uptake of nitrate in barley. 1. Kinetics of $^{13}NO_3^-$ influx. *Plant Physiol.* 93: 1426-1432.

Siddiqi, M. Y., Glass, A. D. M. and Ruth, T. J. (1991) Studies of the uptake of nitrate in barley. 3. Compartmentation of $NO_3^-$. *J. Exp. Bot.* 42: 1455-1463.

Siebrecht, S., Mäck, G. and Tischner, R. (1995) Function and contribution of the root tip in the induction of $NO_3^-$, uptake along the barley root axis. *J. Exp. Bot.* 46: 1669-1676.

Smart, D. R. and Bloom, A. J. (1988) Kinetics of ammonium and nitrate uptake among wild and cultivated tomatoes. *Oecologia* 73: 336-340.

Smart, D. R. and Bloom, A. J. (1993) Relationships between the kinetics of $NH_4^+$ and $NO_3^-$ absoption and growth in cultivated tomato (*Lycopersicon esculentum* Mill. cv T-5). *Plant Cell Environ.* 16: 259-267.

Smart, D. R., Ferro, A., Ritchie, K. and Bugbee, B. G. (1995) On the use of antibiotics to reduce rhizoplane microbial populations in root physiology and ecology investigations. *Physiol. Plant.* 95: 533-540.

Smethurst, P. J. and Nambiar, E. K. S. (1990) Effects of slash and litter management on fluxes of nitrogen and tree growth in a young *Pinus radiata* plantation. *Can. J. For. Res.* 20: 1498-1507.

Smethurst, P. J. and Comerford, N. B. (1993) Simulating nutrient uptake by single or competing and contrasting root systems. *Soil Sci. Soc. Am. J.* 57: 1361-1367.

Smethurst, P. J., Herbert, A. M. and Ballard, L. M. (1997) A paste method for estimating concentrations of ammonium, nitrate, and phosphate in soil solution. *Aust. J. Soil Res.* 35: 209-225.

Smirnoff, N. and Stewart, G. R. (1985) Nitrate assimilation and translocation by higher plants: comparative physiology and ecological consequences. *Physiol. Plant.* 64: 133-140.

Smith, F. A. and Walker, N. A. (1978) Entry of methylammonium and ammonium ions into *Chara* internodal cells. *J. Exp. Bot.* 29: 107-120.

Solomonson, L. P. and Barber, M. J. (1990) Assimilatory nitrate reductase: functional properties and regulation. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 41: 225-253.

Stewart, G. R., Mann, A. F. and Fentem, P. A. (1980) Enzymes of glutamte formation: glutamate dehydrogenase, glutamine synthetase, and glutamate synthase. *In* The biochemistry of plants, Vol. 5. Edited by Miflin, B. J. pp. 271-327. Academic Press, New York.

Swiader, J. M. and Freiji, F. G. (1996) Characterizing nitrate uptake in lettuce using very-sensitive ion chromatography. *J. Plant Nutr.* 19: 15-27.

Tennant, D. (1975) A test of a modified line intersect method of estimating root length. *Journal of Ecology* 63: 995-1001.

Teyker, R. H., Jackson, W. A., Volk, R. J. and Moll, R. H. (1988) Exogenous $^{15}NO_3^-$-influx and endogenous $^{14}NO_3^-$-efflux by two maize (*Zea mays* L.) inbreds during nitrogen deprivation. *Plant Physiol.* 86: 778-781.

Thibaud, J. B. and Grignon, C. (1981) Mechanism of nitrate uptake in corn roots. *Plant Sci. Lett.* 22: 279-289.

Touraine, B., Clarkson, D. T. and Muller, B. (1994) Regulation of nitrate uptake at the whole plant level. *In* A Whole Plant Perspective on Carbon-Nitrogen Interactions. Edited by Roy, J. and Garnier, E. pp. 11-30. SPD Academic Publishing, The Hague.

Tsay, Y., Schroeder, J. I., Feldmann, K. A. and Crawford, N. M. (1993) The herbicide sensitivity gene CHL1 of *Arabidopsis* encodes a nitrate-inducible nitate transporter. *Cell* 72: 705-713.

Turnbull, M. H., Goodall, R. and Stewart, G. R. (1995) The impact of mycorrhizal colonization upon nitrogen source utilization and metabolism in seedlings of *Eucalyptus grandis* Hill ex Maiden and *Eucalyptus maculata* Hook. *Plant Cell Environ.* 18: 1386-1394.

Ullrich, W. R. (1992) Transport of nitrate and ammonium through plant membranes. *In* Nitrogen metabolism of plants. Edited by Mengel, K. and Pilbeam, D. J. pp. 121-137. Oxford University Press, Oxford.

Ullrich, W. R., Larsson, M., Larsson, C.-M., Lesch, S. and Novacky, A. (1984) Ammonium uptake in *Lemna gibba* G 1, related membrane potential changes and inhibition of anion uptake. *Physiol. Plant.* 61: 369-376.

van Beusichem, M. L., Kirkby, E. A. and Baas, R. (1988) Influence of nitrate and ammonium nutrition on the uptake, assimilation, and distribution of nutrients in *Ricinus communus*. *Plant Physiol.* 86: 914-921.

Van Rees, K. J. C. (1994) Michaelis-Menten kinetics: calculation and use in nutrient uptake models. *N. Z. J. For. Sci.* 24: 226-233.

Van Rees, K. J. C., Comerford, N. B. and Mcfee, W. W. (1990) Modelling potassium uptake by slash pine seedlings from low-potassium-supplying soils. *Soil Sci. Soc. Am. J.* 54: 1413-1421.

Vessey, J. K., Henry, L. T., Chaillou, S. and Raper, C. D. J. (1990) Root-zone acidity affects relative uptake of nitrate and ammonium from mixed nitrogen sources. *J. Plant Nutr.* 13: 95-116.

Walker, D. J., Smith, S. J. and Miller, A. J. (1995) Simultaneous measurement of intracellular pH and $K^+$ or $NO_3^-$ in barley root cells using triple-barrelled, ion-selective microelectrodes. *Plant Physiol.* 108: 743-751.

Walker, N. A. and Pitman, M. G. (1976) Measurement of fluxes across membranes. *In* Encyclopedia of Plant Physiology, Vol. 2. Edited by Luttge, U. and Pitman, M. G. pp. 93-126. Springer-Verlag, Berlin.

Walker, N. A., Beilby, M. J. and Smith, F. A. (1979a) Amine uniport at the plasmalemma of Charophyte cells. I. Current-voltage curves, saturation kinetics, and effects of unstirred layers. *J. Membrane Biol.* 49: 21-55.

Walker, N. A., Smith, F. A. and Beilby, M. J. (1979b) Amine uniport at the plasmalemma of Charophyte cells. II. Ratio of matter to charge transported and permeability of free base. *J. Membrane Biol.* 49: 283-296.

Wang, M. Y., Siddiqi, M. Y., Ruth, T. J. and Glass, A. D. M. (1993a) Ammonium uptake by rice roots. 1. Fluxes and subcellular distribution of $^{13}NH_4^+$. *Plant Physiol.* 103: 1249-1258.

Wang, M. Y., Siddiqi, M. Y., Ruth, T. J. and Glass, A. D. M. (1993b) Ammonium uptake by rice roots. 2. Kinetics of $^{13}NH_4^+$ influx across the plasmalemma. *Plant Physiol.* 103: 1259-1267.

Wang, M. Y., Glass, A. D. M., Shaff, J. E. and Kochian, L. V. (1994) Ammonium uptake by rice roots. 3. Electrophysiology. *Plant Physiol.* 104: 899-906.

Wang, X. J., Smethurst, P. J. and Holz, G. K. (1996) Nitrogen mineralization indicies in ferrosols under eucalypt plantations of north-western Tasmania: association with previous land use. *Aust. J. Soil Res.* 34: 925-935.

Warncke, D. D. and Barber, S. A. (1974) Nitrate uptake effectiveness of four plant species. *J. Environ. Qual.* 3: 28-30.

Watt, M. P. and Cresswell, C. F. (1987) A comparison between the utilisation of storage protein and exogenous nitrate during seedling establishment in *Zea mays* L. *Plant Cell Environ.* 10: 327-332.

Wieneke, J. (1992) Nitrate influx in squash seedlings measured with $^{13}N$. *J. Plant Nutr.* 15: 99-124.

Wild, A., Woodhouse, P. J. and Hopper, M. J. (1979) A comparison between the uptake of potassium by plants from solutions of constant potassium concentration and during depletion. *J. Exp. Bot.* 30: 697-704.

Zobel, R. W., Kochian, L. V. and Toulemonde, T. G. (1992) Plant root systems. *In* Proceedings of the Roots of Plant Nutrition Conference. Edited by Reetz, H. F. J. pp. 30-40.